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EDITORIAL

Current dichotomy between traditional molecular biological and omic research in cancer biology and pharmacology

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Abstract

There is currently a split within the cancer research community between traditional molecular biological hypothesis-driven and the more recent "omic" forms or research. While the molecular biological approach employs the tried and true single alteration-single response formulations of experimentation, the omic employs broad-based assay or sample collection approaches that generate large volumes of data. How to integrate the benefits of these two approaches in an efficient and productive fashion remains an outstanding issue. Ideally, one would merge the understandability, exactness, simplicity, and testability of the molecular biological approach, with the larger amounts of data, simultaneous consideration of multiple alterations, consideration of genes both of known interest along with the novel, cross-sample comparisons among cell lines and patient samples, and consideration of directed questions while simultaneously gaining exposure to the novel provided by the omic approach. While at the current time integration of the two disciplines remains problematic, attempts to do so are ongoing, and will be necessary for the understanding of the large cell line screens including the Developmental Therapeutics Program's NCI-60, the Broad Institute's Cancer Cell Line Encyclopedia, and the Wellcome Trust Sanger Institute's Cancer Genome Project, as well as the the Cancer Genome Atlas clinical samples project. Going forward there is significant benefit to be had from the integration of the molecular biological and the omic forms or research, with the desired goal being improved translational understanding and application.

Key words: Omic; Molecular biology; Pharmacology; Cancer; Integration

Core tip: This editorial describes the current split in approach, required expertise, and interpretation between the traditional molecular biological field, and the more recent "omic" approaches to cancer biology and pharmacology. The advantages and limitations of each of these disciplines are discussed and contrasted, highlighting their opposing approaches and mentalities.

The necessity of their efficient integration for the purpose of interpreting both cell line and clinical sample data is argued, especially when trying to project translationally into the clinic.

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PROBLEM OF INTEGRATION OF TRADITIONAL MOLECULAR BIOLOGICAL *VS* "OMIC" RESEARCH

The integration of the traditional molecular biological hypothesis-driven approach with the more recent "omic" forms or research for the purpose of providing translational insight is perhaps the premiere problem for cancer researchers today. How one views these two disparate forms of data impacts both the design and interpretation of biological and molecular pharmacological studies, and subsequently their prospective translational application. However, the two disciplines are by their nature in many ways mirror image opposites of one another, each with their own culture, assumptions, and requirements of expertise. This divergence has in the past, and continues at present to be an impediment to their successful merging.

MOLECULAR BIOLOGICAL APPROACH

The molecular biological approach to research has been dominant for years. It has provided innumerable contributions in the fields of biology, molecular biology, pharmacology, and cancer[1-3]. The mindset of those in the field is rooted in their training, in which questions are ideally distilled down to single alteration-single response formulations that are addressed at the bench experientially. This approach typically requires years of carefully constructed, sequential, narrowly focused studies to explore, confirm, or repudiate their hypothesis. Addressing questions in this fashion allows one to provide quantitative assessments regarding the influence of a specific change on an outcome. The advantages of this approach include understandability, exactness, simplicity, and testability. Typically, improved understanding of one aspect of a pathway also generates testable hypothesis regarding up or downstream events.

However, the use of isogenic systems to focus on specific responses also has important limitations. As molecular events typically occur within the context of pathways, influential events that might occur either upstream or downstream within the salient pathway are typically ignored. Of course, the more complex integration of influences from disparate pathways is also left out. If some single or small number of cell lines is being used to carry out the tests, then the results may be specific for those cell lines used, and less informative in other settings with significant variation. As one tries to apply these results translationally, one immediately encounters the inherent limitation of patients not being isogenic systems. For this reason, to propose that understanding either a patients cancer, or predicting their pharmacological response in the clinic can be successfully done based on an one-gene or one-molecular change type of analysis is likely to provide at best transient insight and benefit, in addition to being the exception to the (more complex) rule. A specific example of this using a dominant molecular event is provided by the BRAF V600E mutation, which provides a useful indicator for efficacious response to vemurafenib in melanoma[4,5]. However, even this unusually robust indication is typically short-lived in its usefulness, as alterations in the tumors undergoing treatment with BRAF inhibitors limit its affective treatment window to some period of months, generally followed by recurrence, often at the same locations[6].

"OMIC" APPROACH

The omic approaches to research, meanwhile, have their own set of advantages and disadvantages. On the positive side, data generated using technology such as array comparative genomic hybridization, transcript microarrays, mass spectrophotometrical proteomic analysis, exome sequencing, cell line screens, or broad spectrum patient sample compendiums view things in the broader context[7-13]. These more inclusive approaches provide the advantage of generating much larger amounts of usable data, allowing the consideration of multiple alterations simultaneously, both in those genes that one might expect to be altered, as well as in those whose involvement is completely unexpected. When the studies include multiple cell lines or patient samples, they also allow cross-sample comparisons to be made. This, of course, allows one to ask directed questions, while simultaneously making novel and potentially important discoveries and observations in totally unexpected areas. A single well-designed omic project can and does typically yield multiple potentially important observations and hypothesis, due to the large amount of data generated.

Unfortunately, there are multiple disadvantages inherent in these approaches as well. By their nature, the omic forms of data necessitate new forms of expertise just to process, and provide basic interpretation and access. These forms of expertise include computer science, statistics, mathematics, and more recently, bioinformatics. When added to the need to understand the results in the context of biology, including the detailed implications of the specific molecular alterations found, both the individual researcher as well the field in general are presented with the need for combinations of cross-disciplinary expertise that are rarely found. In the design

and implementation phase of studies, significant care needs to be taken with issues of quality control and reproducibility. This is necessary to assure the ability to meaningfully compare and interpret data across numerous samples harvested at different times, either from the clinic or cell lines. What cell line or clinical sample to select, their number, how they are handled, and what assay types to perform are all central considerations. For pharmacological studies, which compounds or drugs are selected, their number and type, the conditions under which they are used, and assay type are all key. Once the data assessment and interpretation phase is entered, there are multiple algorithmic approaches that may be used, with their choice being influenced by the data type, the question being asked, and the expertise and biases of the researcher. Correlations, linear regressions, classical statistics, information-theoretic algorithms, and machine learning all have contributions to make in the handling and interpretation of this data[4,14-20]. Additional complexity is then added as multiple forms of data are integrated[21-26]. Finally, algorithmic integration of biological knowledge into the mathematical approach is likely necessary, although this field is in its infancy[27,28].

MOVING FORWARD WITH THE INTEGRATION OF TRADITIONAL MOLECULAR BIOLOGICAL *VS* "OMIC" RESEARCH

So at the current time, integration of the molecular biological and omic disciplines is problematic. Increasing that tension is that the research community as it is constituted today, both at the bench and editorially, is dominated by those with traditional molecular biological training and understanding. This has lead to some reluctance to either accept or understand the omic forms of research. It has even been proposed that the large-scale omic projects are jeopardizing progress in traditional molecular biology due to competition for the research dollar[29].

However, attempts are ongoing throughout the research community to better interpret, integrate, and apply both these forms of data simultaneously[30]. Success may be had by starting with experimental data, and expanding its interpretation by overlaying omic data. This was done in the study of the affect of DNA methylation on E-cadherin expression using standard experimental approaches, and then assessing its influence in the context of multiple regulatory parameters using omic data[31,32]. Conversely, one may start with omic data, and verify its implications with standard experimental approach. This was done with the omic observation that SLFN11 transcript levels had a strong correlation to several drug activities, followed by the use of experimental approaches to prove its causality[33,34].

Both the molecular biological and omic forms of research will be necessary in order to interpret the results of the large cell line screens, including the Developmental Therapeutics Program's NCI-60, the Broad Institute's Cancer Cell Line Encyclopedia, and the Wellcome Trust Sanger Institute's Cancer Genome Project[10-12]. These screens are designed to provide the omic basis for improving the understanding of molecular pharmacology in cancer from the cell line level. Omic analysis has already provided multiple potentially important associations from these databases, including: (1) the association of MEK inhibitor efficacy with AHR expression in NRAS mutant cell lines; (2) a potential affect on the MET inhibitor PHA665752 by amplifications in MET; (3) sensitivity to PARP inhibitors in EWS-FLI1 translocation-containing cells; and (4) the activities of the DNA-damaging bleomycin, zorbamycin, and peplomycin with ATAD5 mutations[4,5,35,36]. All of these omic associations will require traditional molecular biological experimental follow-up to verify or disprove whether they are causal. All insights gained from both the molecular pharmacological and omic approaches will be both useful and necessary for understanding the cells phenotypic differences and establishing a solid basis for drawling inferences. The cell line screens will certainly continue to provide hypotheses and useful study cases going forward. An example of this is the melanoma line LOXIMVI, which while containing the well studied BRAF V600E mutation, still has reduced sensitivity to vemurafenib when compared to the other cell lines containing the mutation, and is thus a potentially useful study case for patient relapse or resistance to that drug.

As one projects to patient samples, such as those found in The Cancer Genome Atlas (TCGA) both molecular biological and omic forms of research will again be necessary as one attempts to provide interpretation[13]. TCGA is designed to provide a base for omic analysis of clinical samples, providing data on some about 9939 patients from 33 cancer types. It provides both molecular and patient therapeutic information. Omic analysis of this data has already provided multiple potentially important associations, including: (1) targets for pharmacological intervention in squamous cell cancer including FAT1, MLL2, TGFRBR2, HLA-A, and NFE212; (2) a potentially clinically relevant association between elevated levels of CX43 in glioblastoma tumor samples and temozolomide resistance; and (3) multiple FDA-approved drug targets of metabolic vulnerabilities[37-39]. As was the case for the cell line screens, these omic associations will require traditional experimental follow-up to verify or disprove their causality.

Going forward, considering the daunting set of challenges facing the researcher, it should be clear that all insights derived from both the traditional molecular biological and omic approaches will be both desirable and necessary to make sense of the complex and overlapping challenges that exist. As progress is made in these areas, one hopes that making patient treatment decisions based on that patient's complex

molecular profile will become the norm. An integrated vision for the molecular biological and omic approaches will be helpful if not necessary to that end.

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EDITORIAL

Current role of spacers for prostate cancer radiotherapy

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Abstract

Radiotherapy is an established curative treatment method for prostate cancer. Optimal tumor control rates can only be achieved with high local doses, associated with a considerable risk of rectal toxicity. Apart from already widely adapted technical advances, as intensity-modulated radiation therapy, the application of spacers placed between the prostate and rectum has been increasingly used in the last years. Biodegradable spacers, including hydrogel, hyaluronic acid, collagen or an implantable balloon, can be injected or inserted in a short procedure under transrectal ultrasound guidance *via* a transperineal approach. A distance of about 1.0-1.5 cm is usually achieved between the rectum and prostate, excluding the rectal wall from the high isodoses. Several studies have shown well tolerated injection procedures and treatments. Apart from considerable reduction of rectal irradiation, a prospective randomized trial demonstrated a reduction of rectal toxicity after hydrogel injection in men undergoing prostate image-guided intensity-modulated radiation therapy. The results are encouraging for continuing evaluation in dose escalation, hypofractionation, stereotactic radiotherapy or re-irradiation trials in the future.

Key words: External-beam radiotherapy; Intensity-modulated radiotherapy; Brachytherapy; Spacer; Hydrogel; Biodegradable balloon; Hyaluronic acid; Collagen; Prostate cancer; Toxicity

Core tip: Radiotherapy is widely used for the treatment of prostate cancer. Technical advances allow improved tumor control with increasing prescription doses, but rectal wall is known to be a dose-limiting organ. A new method that has been increasingly used in the last years is the application of a biodegradable spacer to increase the distance between the prostate and rectal wall. Clinical studies, including a prospective randomized trial, have reported considerable dosimetric advantages for the rectum, well tolerated insertion procedures and radiotherapy treatments.

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INTRODUCTION

Radiotherapy is an established curative treatment method for prostate cancer. Prospective randomized trials evaluating dose escalation have consistently shown significantly higher biochemical control rates for higher doses. However, significantly higher rectal toxicity rates resulted[1]. Rectal toxicity is regarded as the dose-limiting toxicity[2]. Rectal toxicity has been evaluated in a large number of studies and dose-volume correlations have been clearly established[3-5]. Apart from higher dose and larger volumes within specific isodoses, risk factors for toxicity after radiotherapy include history of prior abdominal surgery, advanced age, diabetes mellitus, concomitant use of androgen deprivation, hemorrhoids, and inflammatory bowel disease[6].

Most of the randomized dose escalation studies applied three-dimensional conformal techniques[1]. Several further technical advances have been introduced in the last years. Intensity-modulated radiotherapy (IMRT) techniques, currently regarded as a standard for prostate cancer treatment in an increasing number radiation oncology departments, result in improved dose conformality[7]. Image-guided radiotherapy (IGRT) is applied to show the prostate position before or even during each fraction, so that treatment margins and volumes can be reduced[8]. Using these techniques, new concepts as hypofractionated treatments or even stereotactic radiotherapy treatments have been introduced in the past, resulting in a considerable shortening of the external beam radiotherapy (EBRT) treatment period[9].

As the prostate is usually situated without a relevant distance to the rectal wall and EBRT requires safety margins around the prostate of about 4-10 mm (depending on several factors as patient positioning and IGRT method), the anterior rectal wall is always included in the planning target volume and thus the prescription isodose. The insertion of a spacer between the prostate and rectum is an increasingly used method to create a considerable distance between the prostate and rectum and thus exclude the rectum from the high dose volume. A high dose can be delivered safely with adequate margins[10].

OPTIONS

Requirements for a spacer are a well tolerated insertion, a stable position during up to two months of radiotherapy and biodegradation. A spacer should not be allergenic or toxic. Studies in prostate cancer patients evaluated the effects of hyaluronic acid, human collagen, inflatable balloon or hydrogel as different spacer materials[11-14]. Hyaluronic acid is a natural polysaccharide component in connective tissue and extracellular matrix[15]. Human collagen is known from injections into the perineum to treat urinary incontinence[16]. An inflatable biodegradable balloon (PLCL, polylactide-co-ε-caprolactone) has been specifically introduced to be used as a spacer[17]. In the past absorbable polyethylene glycol (PEG) hydrogels have been applied in surgical procedures as lung, dural and vascular sealants[14]. Hydrogels are injected as liquids and polymerize in situ within < 10 s following the mixture of two precursor solutions.

A transperineal approach with transrectal ultrasound (TRUS) guidance is used for spacer implantation/injection under local, spinal or light general anaesthesia. The actually selected anaesthesia will be chosen depending on the procedures planned (length and depth needed for incision, gold marker implantation, brachytherapy) and the respective local protocol. The approach is well known from prostate brachytherapy or gold marker implantation for IGRT. A needle is placed about 1-2cm anteriorly to the TRUS probe and forwarded to the prostatic apex. Prior hydrodissection facilitates spacer insertion. The spacer must be positioned between the rectal wall and the Denonvilliers' fascia[18]. In a series including 243 prostatectomy specimens, Villers *et al*[19] reported that prostate cancer invaded Denonvilliers' fascia in 19% of cases, but no patients presented a tumour progression through the full thickness of the fascia. Thus, the risk of tumour cell displacement can be regarded to be minimal.

Prada *et al*[13] performed hyaluronic acid injections without hydrodissection. Hydrogel or human collagen are injected following prior hydrodissection - the same-18 gauge spinal needle is used for hydrodissection and spacer injection[12,18]. Injection of fluid spacers is less invasive in comparison to the balloon implantation. However, a balloon can be deflated and repositioned if required.

An incision of 3-5 mm is required before implantation of a biodegradable balloon. The incision allows the dilatator and the introducer sheath to be inserted into the perineum. The dilatator is advanced towards the prostate base over the needle and the needle removed subsequently. The introducer sheath acts as a working channel for the introduction of the balloon. The balloon is filled with warm saline and sealed with a biodegradable plug following a full inflation[11,20].

TREATMENT PLANNING

As demonstrated in several studies, the injection or insertion of a spacer results in a distance of about 1.0-1.5 cm between the prostate and rectum, so that the rectal wall and planning target volume do not overlap[11-13,21]. The largest study included 100 patients after hydrogel injection[22]. A higher injected volume can result in a larger separation. Hydrogel is usually inserted in standardized 10-15 mL systems[14,22]. Comparably to the hydrogel studies, a mean separation of 12.7 mm was achieved with 20 mL human collagen injections in a pilot study[12]. The inflation of a balloon with nearly 20 mL of saline can result in mean prostate-rectum separation > 1.5 cm[11]. Though different injection volumes have not been compared in studies, an increasing volume can be potentially associated with toxicity related to

the pressure on the rectal wall or even the prostate. A volume of 10-15 mL and a resulting distance of 1.0-1.5 cm appear to be very effective and well tolerable for the patients[11-14,22].

This separation results in a considerable dosimetric advantage for the rectum. In EBRT studies, relative rectal wall volume reductions of > 70% within the 90% isodose levels have been shown comparing treatment plans prior and following spacer insertion, *i.e.*, only < 5% of rectal volume is included in the 70Gy isodose when a prescription dose of 78-79Gy is used[11,15,21]. Guidelines recommend to limit this volume to 20%[23], so that these recommendations can be met without problems. Thus, to reach an optimal dose distribution, treatment planning after spacer insertion must include much lower objectives for the rectum. The information from the dose-volume histogram indicates a low risk of rectal toxicity. On the other hand, it implicates the potential for safe delivery of new hypofractionated and stereotactic treatments or re-irradiation concepts without a relevant risk of higher grade rectal toxicity.

CLINICAL EXPERIENCE

Spacer studies have been reported after several different treatment concepts, as low-dose rate[24] and high-dose rate (HDR) brachytherapy[22,25] with or without additional EBRT, hypofractionated EBRT concepts[26] or proton and heavy ion concepts[27,28]. Rare spacer-related complications have been reported in the literature, as focal rectal necrosis or ulceration as a result of unintentional injection of hydrogel into the rectal wall or urinary retention, usually resolving within a short time[14].

Vanneste *et al*[29] calculated the cost-effectiveness of treating prostate cancer patients with and without a spacer, using a decision-analytic Markov model. According to the Dutch health costs, the spacer was found to be cost-effective for prostate cancer patients due to less severe toxicity and a reduction in treatment costs associated with side effects.

Taking into account a lack of long-term clinical experience with spacers, radiobiological models can be used to estimate long-term toxicity. They correlate prior data from the treatment plan and long-term toxicity[30]. Mean normal tissue complication probability (NTCP) for severe proctitis, necrosis, fistula or ≥ grade 2 rectal bleeding was found to be reduced by ≥ 50% comparing data before *vs* after hydrogel spacer injection. A clear advantage was shown for conventional and IMRT techniques[31].

The vast majority of published clinical studies have used hyaluronic acid or hydrogel. Studies with hyaluronic acid included smaller patient groups. Prada *et al*[25] did not observe grade 2 or higher toxicity after HDR brachytherapy as monotherapy (single 19Gy fraction) in an analysis of 40 patients and a median follow-up of 18 mo. Chapet *et al*[26] reported the acute toxicity of a hypofractionated IMRT with 3.1Gy fractions up to 62Gy total dose in 36 patients, without any grade 2 or higher toxicities.

PEG hydrogel stability during treatment has been shown in studies, so that a constant prostate-rectum separation can be expected[32]. Hydrogel starts to liquify about 3 mo after injection, is absorbed within about 6 mo and cleared *via* renal filtration. Prostate position variability is similar with or without hydrogel, so that IGRT is still required with a spacer to keep safety margins small. However, in contrast to patients without a spacer, larger posterior displacements were not found with a spacer[32].

A learning curve has been reported in a study including 64 patients, showing an increasingly symmetrical hydrogel distribution and significantly larger prostate-rectum distances with the same hydrogel volume. As a consequence, an improved dosimetric rectum protection and smaller acute bowel quality of life changes resulted[10].

Gastrointestinal toxicity (GI) was analyzed in a study including 48 patients in a multi-institutional prospective study. Grade 2 acute GI toxicity was reported in only 12% of patients (no grade 3-4 toxicity). Grade 1 late GI toxicity was found in 7% of patients within 12 mo after treatment (corresponding to two patients, one of them with grade 1 at baseline; no patients with grade 2-4 toxicity)[14].

In a prospective randomized multicenter study 222 patients were randomized between a treatment with and without hydrogel (149 patients with and 73 without spacer). Patients were treated after fiducial marker placement (IGRT) with 1.8Gy fractions up to a total dose of 79.2Gy, using an IMRT technique. There were no device-related adverse events, rectal perforations, serious bleedings or infections within either groups[21]. Mean rectal volume within the 70Gy isodose was reduced from 12% to 3%. As also reported in a prior case control study[33], similar acute rectal toxicity was observed in both patient groups. However, a significant reduction in late (3-15 mo) rectal toxicity in the spacer group was observed (2% *vs* 7%). There was no late rectal toxicity greater than grade 1 in the spacer group. At 15 mo, 12% and 21% of spacer and control patients experienced 10-point declines in bowel quality of life (EPIC questionnaire, Expanded Prostate Cancer Index Composite)[21].

CONCLUSION

The number of published studies reporting clinical data with spacer materials for prostate cancer radiotherapy is increasing. Hydrogel, hyaluronic acid, collagen or an implantable balloon, can be injected or inserted under transrectal ultrasound guidance. Most studies, including several studies with more than 50 patients treated with a spacer and a recently published prospective randomized study, evaluate the effects of a hydrogel spacer (Figure 1). A distance of about 1.0-1.5 cm is usually achieved between the prostate and rectum,

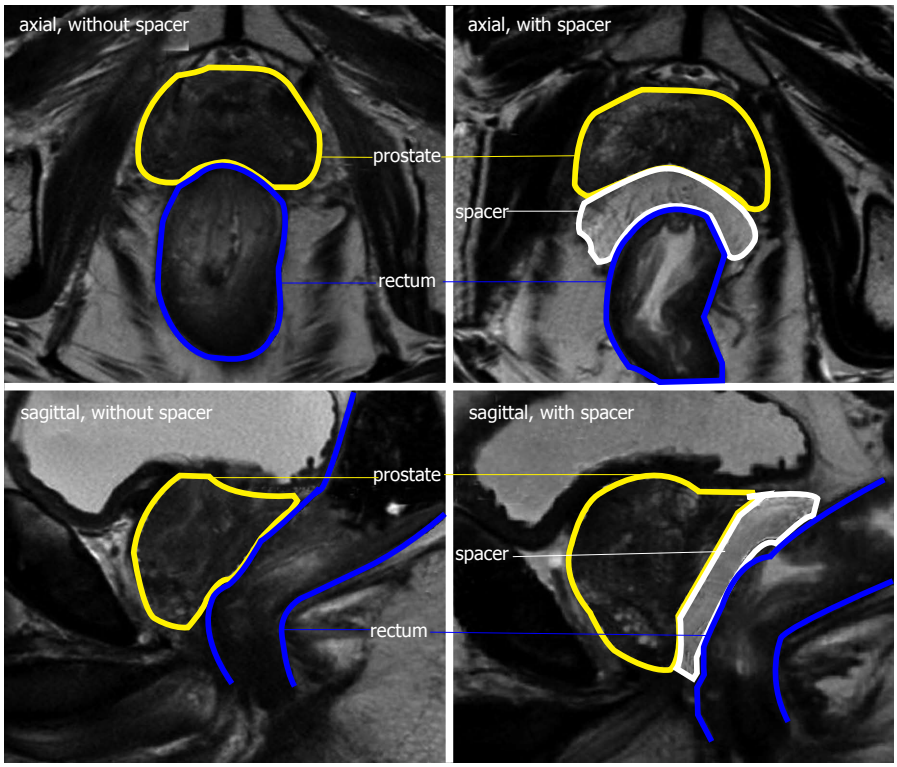

Figure 1 T2 weighted magnetic resonance imaging without (left) and with (right) a hydrogel spacer. Spacer hyperintense, resulting in > 1 cm separation between prostate and rectum.

excluding the rectal wall from high isodoses. Procedure or spacer related complications are rare and treatments well tolerated. Reduced late toxicity rates have been shown in a prospective randomized study. Long-term results with a follow-up > 2 years are not available yet. Presently available results are encouraging for the design of further clinical studies.

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EDITORIAL

Neuroendocrine tumors resistant to mammalian target of rapamycin inhibitors: A difficult conversion from biology to the clinic

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Abstract

Deregulation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) - mammalian target of rapamycin (mTOR) signaling pathway is one of the most commonly-involved pathways in tumorigenesis. It has also been reported as altered in neuroendocrine tumors (NETs). mTOR inhibitors used in clinical practice are derived from rapamycin, an anti-cancer agent also used as an immunosuppressor after organ transplantation. Everolimus and temsirolimus are the two rapamycin-derived mTOR inhibitors used in NETs. Notably everolimus has been approved in advanced progressive well/moderately-differentiated pancreatic NETs (pNETs). It inhibits specifically the mTORC1 subunit of mTOR, not interacting with mTORC2. Although everolimus produced a significant prolongation of progression-free survival a number of patients with pNETs do not benefit from the drug due to early or late progression. Two supposed mechanisms of resistance to mTOR inhibitors are Akt and PI3K activation, by means of mTORC2 and insulin growth factor (IGF) - IGF receptor signaling, respectively. BEZ235 is a multi-targeted inhibitor binding to PI3K, mTORC1 and mTORC2, therefore potentially turning off all the supposed molecular targets of resistance to everolimus. The two clinical trials designed in pNETs were stopped early due to unmet statistical endpoint and the global clinical development of BEZ235 was also halted. Tolerability of this drug was challenging and conditioned the feasibility of therapy. The BEZ experience is an example of the huge difference between the preclinical and clinical setting and prompts us to pay more attention to the phase Ⅰ step of clinical development and the design of phase Ⅱ clinical trials.

Key words: Everolimus; BEZ235; Mammalian target of rapamycin; Phosphoinositide 3-kinase; Mammalian target of rapamycin C; Resistance; Mammalian target of rapamycin inhibitor

Core tip: Although everolimus significantly prolongs progression-free survival in patients with advanced

pancreatic neuroendocrine tumors (NETs), some patients are refractory or progress early after an initial response. Mammalian target of rapamycin (mTOR) C2 and insulin growth factor (IGF) - IGF receptor signaling can mediate two supposed mechanisms of resistance to everolimus. BEZ235 is a multitargeted inhibitor binding to phosphoinositide 3-kinase, mTORC1 and mTORC2, therefore potentially turning off all the supposed molecular targets of resistance to everolimus. The two clinical trials designed in pancreatic NETs were stopped early due to unmet statistical endpoint and the global clinical development of BEZ235 was halted. Challenging tolerability probably conditioned the results. The BEZ experience is an example of the huge difference between preclinical and clinical setting and prompts us to pay more attention to the phase Ⅰ step of clinical development and the design of higher-phase trials.

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MAMMALIAN TARGET OF RAPAMYCIN PATHWAY

The mammalian target of rapamycin (mTOR) is a sort of intracellular metabolic switch, that physiologically regulates growth, proliferation and survival of normal cells integrating growth factors and nutrient signals[1]. It is an intracellular serine/threonine kinase activated by two main upstream factors, namely phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt). This in turn activates downstream factors, including the ribosomal protein S6K and eukaryotic translation initiation factor 4E binding protein (4EBP-1). Based on the interaction of mTOR with other proteins, two functionally distinct subunits exist, mTORC1 and mTORC2; among others mTORC1 includes the regulatory-associated protein of mTOR, whereas mTORC2 includes the rapamycin-insensitive companion of mTOR. Activated mTORC1 activates in turn $p70^{S6K}$, the kinase that phosphorylates the ribosomal protein S6, finally inducing protein synthesis. Activation of mTORC1 and S6K inhibits the tyrosine phosphorylation and signaling functions of insulin receptor substrates (IRS-1) through a negative feedback mechanism, resulting in the attenuation of PI3K–Akt signaling. Activated mTOR leads to protein synthesis also through 4EBP1 activation, inducing translation.

One of the main upstream stimulating factors of mTOR is the insulin-like growth factor (IGF) and its receptor (IGFR), activated by IRS-1; whereas phosphatase and tensin homolog, tuberous sclerosis complex and neurofibromatosis-1 factor are inhibitors of mTOR signaling.

Deregulation of PI3K-Akt-mTOR signaling pathway is one of the most common mechanisms of tumorigenesis[2]. It has been reported as dysregulated also in neuroendocrine tumors (NETs), familial and sporadic[3,4].

mTOR INHIBITORS

The term mTOR derives from rapamycin, which is a macrolide, initially studied as an antifungal and antibiotic agent, known for its immunosuppressant activity, which has also demonstrated antitumor properties. Two derivatives of rapamycin have been used in NETs, everolimus and temsirolimus. Everolimus (RAD001) was approved by the FDA and EMA in progressing well-moderately differentiated pancreatic NETs (pNETs), based on the results of a randomized phase Ⅲ study comparing it with placebo (RADIANT-3 trial).

RESISTANCE TO mTOR INHIBITION

Some patients with pNETs show primary or secondary (acquired) resistance to everolimus. The precise mechanism is unknown, but some hypotheses have been postulated, including Akt activation by means of mTORC2 and IGF1/IGFR signaling activation due to inhibition of the S6K negative feedback[5,6]. On this basis, drugs inhibiting these supposed targets of resistance were preclinically studied.

DUAL INHIBITOR BEZ235

BEZ235 is a potent oral multitargeted inhibitor of all four class Ⅰ PI3K isoforms and the downstream effectors, mTORC1 and mTORC2[7]. In preclinical studies BEZ235 showed clearly higher activity than everolimus in NETs[8-10] and BEZ/everolimus combination was suggested as synergistic[8,9]. Furthermore BEZ235 reversed resistance to other anti-cancer therapies in a variety of tumor cell line[11-13]. However, conducting phase Ⅰ studies with this agent has proved challenging. In the more than 200 patients treated, both by single agent and in combination, in several phase Ⅰ / Ⅰ b studies with different types of tumor, the formulation was changed moving from gelatine capsule to sachet[14]. Furthermore, the schedule was moved from QD (once per day) to BID (twice per day). High intra- and inter-patient pharmacokinetic variability was observed. In spite of these troubling premises, given the impressive preclinical activity, a world BEZ235 clinical development plan was launched for several types of tumor, including prostate, breast, renal cancers and pNETs. In pNETs two trials were designed with BEZ235 as single agent: One small multicentre phase Ⅱ trial in pNETs resistant to everolimus and a large randomized phase Ⅱ *vs* everolimus in pNETs not previously treated with mTOR inhibitors. Both were prematurely halted, the former after completion of the first stage with 30 patients

enrolled, due to unmet statistical endpoint, and the latter after randomization of 62 out of the 140 foreseen patients, due to unlikely superiority to everolimus at a first interim analysis of 35 patients[15]. In the phase Ⅱ trial beyond everolimus the initial BEZ235 dose of 400 mg was amended to 300 mg due to intolerable toxicity. This agrees with a recently published phase Ⅰ study of 33 patients with different types of malignancies who received BEZ235 administered twice daily as an oral sachet, where 300 mg BID resulted the recommended dose[14].

The poor tolerability of BEZ235 negatively influenced both studies. Although its toxicity profile was confirmed without evidence of new toxicities, BEZ235 was less well tolerated than everolimus in the randomized study; furthermore in both studies a high percentage of adverse events led to frequent treatment discontinuation, in particular 39% in the BEZ arm of the randomized study (*vs* 16% for everolimus) and 36% in the phase Ⅱ stage Ⅰ study. Based on this experience, further clinical investigation of BEZ235 in cancer was halted.

CONCLUSION

This is an example of the huge difference that sometimes exists between bench and bedside. Why would a drug such as BEZ235, which binds to the potentially correct targets for overcoming mTOR inhibition resistance and which is highly effective in preclinical investigations, meet with failure in clinical trials? A number of reasons may be advanced, both tumor-related and drug-related. Of course it is possible that the PI3K pathway is not the sole driver of resistance in pNETs and/or that the targets of BEZ235 do not represent the mechanism of activation of the PI3K pathway in some pNETs. However, the difficulty in managing the BEZ235 changeable toxicity in trials beyond phase Ⅰ / Ⅰb strongly suggests that it is not only a matter of level of dose and therefore that the maximum tolerated dose concept is not suitable for all drugs. Other areas, including transportomics and metabolomics, which can strictly influence the tolerability of a drug, should be investigated. On the one hand, BEZ235 has a particular oral formulation susceptible to variable absorption while on the other hand its metabolism depends on CYP3A4, CYP1A2 and aldehyde oxidase activity.

Finally, the BEZ235 experience has taught that multidisciplinarity could be useful for planning an anti-cancer agent clinical investigation, with clinicians dedicated to the specific tumor area and with pharmacologists who should work in close collaboration with phase Ⅰ trial clinical researchers.

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EDITORIAL

Inorganic phosphate in the development and treatment of cancer: A Janus Bifrons?

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Abstract

Inorganic phosphate (Pi) is an essential nutrient to living organisms. It is required as a component of the energy metabolism, kinase/phosphatase signaling and in the formation and function of lipids, carbohydrates and nucleic acids and, at systemic level, it plays a key role for normal skeletal and dentin mineralization. Pi represents an abundant dietary element and its intestinal absorption is efficient, minimally regulated and typically extends to approximately 70%. Maintenance of proper Pi homeostasis is a critical event and serum Pi level is maintained within a narrow range through an elaborate network of humoral interactions and feedback loops involving intestine, kidney, parathyroid gland and bone, and depends on the activity of a number of hormones, including parathyroid hormone, 1,25-dihydroxy vitamin D, and fibroblast growth factor 23 as major regulators of Pi homeostasis. Notably, Pi intake seemingly continues to increase as a consequence of chronic high-phosphorus (P) diets deriving from the growing consumption of highly processed foods, especially restaurant meals, fast foods, and convenience foods. Several recent reports have generated significant associations between high-P intake or high-serum Pi concentration and morbidity and mortality. Many chronic diseases, including cardiovascular diseases, obesity and even cancer have been proposed to be associated with high-P intakes and high-serum Pi concentrations. On the other hand, there is also evidence that Pi can have antiproliferative effects on some cancer cell types, depending on cell status and genetic background and achieve additive cytotoxic effects when combined with doxorubicin, illustrating its potential for clinical applications and suggesting that up-regulating Pi levels at local sites for brief times, might contribute to the development of novel and cheap modalities for therapeutic intervention in some tumours. Overall, the influence of Pi on cell function and the possible relationship to cancer have to be fully understood and investigated further.

Key words: Calcium-phosphate nanoparticles; Inorganic phosphate; Cancer; High-phosphorus diets; Phosphorus intake; Doxorubicin; Combination therapy; Naturally occurring molecule; Osteosarcoma

Core tip: Many chronic diseases, including cancer have been proposed to be associated with high-phosphorus intakes and high-serum inorganic phosphate (Pi) concentrations. On the other hand, there is also evidence that Pi can have antiproliferative effects on some cancer cell types, depending on cell status and genetic background and achieve additive cytotoxic effects when combined with doxorubicin, illustrating its potential for clinical applications and suggesting that up-regulating Pi levels at local sites for brief times, might contribute to the development of novel and cheap modalities for therapeutic intervention in some tumors, including triple-negative breast cancer and osteosarcoma.

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INORGANIC PHOSPHATE AND CANCER

One of most important nutrients to living organisms is Inorganic phosphate (Pi). It is required in the ATP formation, kinase/phosphatase signalling and in the synthesis of lipids, carbohydrates and nucleic acids. Furthermore, it plays a key role for normal skeletal and dentin mineralization[1].

Diet represents the main source of Pi intakes, its intestinal absorption is minimally regulated and typically extends to approximately 70%. To maintain Pi levels within a proper range, an elaborate network, including intestine, kidney, parathyroid gland and bone, is involved in a feedback control in which hormones as parathyroid hormone (PTH), 1,25-dihydroxy vitamin D, and fibroblast growth factor 23 (FGF-23) are major regulators of Pi homeostasis[2].

Diets always richer in phosphorus, due to a highly processed food, especially restaurant meals, fast foods, and cheap foods, have increased Pi intake[3,4].

For example, in the United States the consume of phosphorus daily in meals is typically around 1400 mg, as inorganic phosphate (Pi) salts or as a part of organic molecules, that is almost doubled compared to the adult recommended dietary allowance.

The kidney is one of the major regulators of Pi homeostasis and can increase or decrease its capacity to reabsorb Pi; the increased cumulative use of ingredients containing Pi in food processing is now being shown to be potential toxic when it exceeds nutrient needs.

Several recent studies have underlined the relationship between high-Pi intake/high-Pi serum concentration and morbidity and mortality[3,4].

A variety of conditions and diseases, especially cardiovascular diseases, has been spotted in individuals with high-Pi intakes, resulting from chronic high-Pi diets. Other chronic diseases, including type 2 diabetes mellitus, obesity and even cancer have also been proposed to be associated with high-Pi intakes and high-Pi serum concentrations[3-5].

As far as the mechanisms by which high Pi concentrations are linked to tissue damage and/or possibly to influence tumour growth, they are not completely understood and could very likely include a mixture of cell autonomous as well as autocrine, paracrine, and/or endocrine signals.

In particular, although both PTH and FGF-23 are stimulated to decrease the post-meal serum Pi concentration rise, approximately 1 h through the interruption of renal Pi reabsorption, it is hypothesized that if cells are exposed to even a brief high-serum Pi concentration there could be some signal alterations in cell functions leading to negative effects. Moreover, the increase of serum levels of FGF-23 or PTH might be toxic to particular cell types[3,4,6].

Numerous recent studies have reinforced a long-standing hypothesis that there could be a phosphate-sensing mechanism capable of detecting serum and local phosphate variations and of informing the body, the local environment or the individual cell[7,8]. Because of the fact that the intracellular environment is electronegative compared to the extracellular one, the Pi transit into the cell does not happen by simple diffusion, but is mediated by Na^+-coupled Pi cotransporters, which is a regulated event[9]. In addition, Pi is coming out as an essential signalling molecule capable of modifying a lot of cellular functions by varying signal transduction pathways, gene expression and protein levels in many cell types[8,10-12].

It has been shown that high tissue phosphate concentrations increase oxidative stress in endothelial cells[13]. In human vascular smooth muscle cells it has been demonstrated that inorganic phosphate has effects on cell cycle and apoptosis, as well as, in the same cells, the increase of phosphate levels influences cellular and matrix elements promoting calcification[14,15].

Moreover, it has also been supposed that high inorganic phosphate value speeds up senescence process in mouse models[16]. Recent data have confirmed that diets with a high intake of Pi enlarge tumorigenesis in the two-stage skin carcinogenesis model and K-ras lung cancer model in mice[17,18].

In addition, inorganic phosphate has been demonstrated to promote the activation of distinct pathways like ERK1/2 and Akt kinases, as well as it stimulates cell growth in specific cell types, such as preosteoblastic MC3T3-E1 cells, human lung cells, epidermal JB6 cells, proposing Pi as a mitogenic molecule in these cells[17-21].

Recently, a large scale transcriptomics and proteomics research has evidenced that many pro-angiogenic genes and proteins are upregulated by raised Pi levels in preosteoblasts cells[22] as osteopontin (OPN), a secreted cytokine, and forkhead box protein C2 (FOXC2), a

forkhead box transcription factor, both proteins recently associated with tumour angiogenesis. Lately, it has been demonstrated that in cancer cells Pi encourages tube formation and endothelial cells migration in vitro if exposed to elevated extracellular Pi levels, with FOXC2 and OPN as possible proteins involved in this mechanism[23].

Notably, the pro-tumors and proliferative effects of Pi are not possible to extend to all cell types, in fact, it has been related that in MO6-G3 odontoblast-like cells Pi induces apoptosis[23,24].

Previously, in the last years, we published a succession of articles, in which the aim has been to study the effects of elevated Pi on human osteosarcoma cell line U2OS and to know possible molecular mechanisms involved[25-28].

Initially, we demonstrated that inorganic phosphate inhibits cell growth and reduces aggressiveness of human osteosarcoma cell line U2OS, identifying adenylate cyclase, beta3 integrin, Rap1, ERK1/2 as proteins whose expression and function are influenced by Pi[25,26].

Later on, we proved also that Pi is capable of increasing the sensibility of osteosarcoma cells to doxorubicin in a p53-dependent manner and through down-regulation of ERK1/2 pathways[27,28].

More recently, we described initial evidence of a strong antiproliferative action of Pi in MDA-MB-231 cell line, an extremely aggressive human triple negative breast cancer model, enlarging the hypothesis of Pi as a novel signalling molecule capable of modifying the function and survival of specific cell types[11].

As part of our continuing effort to extent the knowledge on the role of inorganic phosphate as a "naturally occurring molecule" acting also as a "sensitizer" to increase the therapeutic index of clinical antitumor drugs, in a current study we describe that Pi induces strongly sensitization to doxorubicin by apoptosis induction in MDA-MB-231. We also show that Pi increases doxorubicin-induced cytotoxicity and that this mechanism involves ERK1/2 and STAT3 down-regulation[29].

It is important to underline that in our studies we use a very low doxorubicin dose (until 0.1 μmol/L) that it is known to be a bearable dose because related to minimal side effects in patients, thus suggesting the possible clinical relevance of this positive pharmacological interaction[30,31].

Latterly, new drug delivery system, called Calcium-phosphate nanoparticles, has been built up. Moreover, it is important to remember that hydroxyapatite nanoparticles release inorganic phosphate and that its retention, most likely, modifies Pi concentration at local sites[32,33].

Furthermore, phosphate is the richest anion in the intracellular environment, with a concentration of 100 mmol/L, so it is easy to find an increase of extracellular Pi as a consequence of cell death induced by chemotherapy.

Maintenance of Pi systemic levels remains a crucial point, because an increase of serum values, even if moderate, and polymorphisms in genes implicated in Pi homeostasis may have effects on ageing process and lifetime[2].

The quantities of inorganic phosphate continue to rise in the diet, in particular way in the western countries, and an increase of the morbidity and mortality in the exposed population has been linked to this habit[3,4].

In Particular, it is known that diet is an environmental element which can be manipulated; it has important consequences on genomics and proteomics functions and it is strongly connected to cancer[34,35].

Inorganic phosphate, as a common dietary element, might modify cells behaviour. However, the possibility that Pi can modify cell functions and its relationship to cancer have to be fully understood and investigated further[36,37].

By the way, the findings that inorganic phosphate, a simple "naturally occurring molecule", can have antiproliferative actions on some cancer cell types, depending on cell status and genetic background (p53, estrogen receptors, caspases expression, *etc.*) and can increase cytotoxic effects when combined with doxorubicin, show its potential for clinical applications, suggesting that up-regulating Pi levels at local sites for brief times might contribute to the development of novel and cheap modalities for therapeutic intervention in some tumors, including triple-negative breast cancer and osteosarcoma.

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EDITORIAL

Sentinel lymph node metastasis after neoadjuvant treatment in breast cancer: Any size matters?

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Abstract

One of the advantages of neoadjuvant chemotherapy (NAC) treatments is its ability to convert patients who need a mastectomy in breast conservative surgery. NAC has also increased the conversion of node positive patients into node negative in around 40% allowing the use of sentinel node biopsy (SLN) in this setting. Timing of SLN biopsy after NAC has been a subject of debate. In patients with clinically node negative before NAC, rates of success and false negative rates of SLN after NAC are similar to those in the adjuvant setting, so SLN after NAC in previous negative axilla has been incorporated in the staging of the axilla. More controversial is its use in patients with positive axillary nodes before NAC who convert to node negative after NAC. Several randomized studies have reported the identification rates and the false negative rates of the SLN after NAC, concordant in the importance of surgical technique. As there is an agreement in the abandon of the immunohistochemistry (IHC) for SLN in the adjuvant setting as SLN IHC detected metastasis appear to have no impact on overall survival, in patients with SLN after NAC the inclusion of isolated tumor cell (ITC) as positive nodes lowers the false negative rates of the technique, suggesting the importance of assessing the SLN by IHC after NAC and considering it as residual disease. Longer follow up is needed to determine the prognostic implications of ITC in the SLN after NAC.

Key words: Sentinel node; Metastasis; Neoadjuvant treatment; Breast cancer

Core tip: One of the advantages of neoadjuvant chemotherapy treatment in breast cancer is to downstage positive axillary nodes to negative. Postneoadjuvant sentinel lymph node (SLN) has been increasingly used and randomized studies in patients with positive axillary nodes who convert to node negative have shown that false negative rates are highly influenced by the surgical technique. Information from these studies has shown that isolated tumor cells in the SLN, when considered as positive nodes, lower false negative rates. Whether any residual disease in the SLNs may have prognostic implications warrants further research.

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RATIONALE FOR NEOADJUVANT TREATMENT IN BREAST CANCER

Neoadjuvant chemotherapy (NAC) is an accepted treatment for locally advanced and early stage breast cancer as it has shown many advantages. It allows *in vivo* determination of an individual tumor's chemosensitivity, it reduces micrometastatic disease and it can downstage tumors, allowing for breast conserving surgery in previously ineligible patients for conserving surgery[1]. Randomized studies have reported rates of downstaging after NAC between 49%-94% and 20%-40% of patients achieve a complete pathologic response[2-6].

There is clear evidence that NAC downstages positive axillary nodes in a proportion of patients. Early studies have shown that NAC can completely clear axillary metastases in approximately 23% of patients with locally advanced breast cancer[6], rates that have increased to 40%-60% with the use of targeted therapies[7]. Axillary complete downstaging after NAC has been correlated with better prognosis and assessment of residual disease after NAC is important not only in determining the prognostic information but also in selecting candidates for further systemic and radiation therapy treatment[7,8].

SENTINEL LYMPH NODE AFTER NEOADJUVANT TREATMENT

Timing of sentinel lymph node (SLN) in breast cancer patients undergoing NAC has been subject of continuous debate. An advantage of performing SLN after NAC is a single surgery and that patients with downstaging axillary nodes after NAC may potentially spared an axillary lymph node dissection (ALND). Most authors have taken the position to do it after NAC[9-14], and results from meta-analysis and prospective studies have reported a success rate of SLN identification after NAC of 90% and rates of false negative around 10.5%[9,10]. In patients with clinically negative axilla, rates of success and false negative rates are similar to those in the adjuvant setting, so SLN after NAC in previous negative axilla has been incorporated in the staging of the axilla[15]. Recently, in a population-based study of SLN before (980 patients) or after NAC (203 patients) in clinically node negative patients of the Netherlands Cancer Registry, the SNL identification rate was higher in the SLN pre NAC group *vs* after NAC (98% *vs* 95%; P = 0.032). Significantly, a lower proportion of patients had a negative SNB pre NAC compared to after NAC. In 67% of patients with SNB after NAC no axillary treatment was given, compared to 55% of the patients with SNB before NAC. The authors conclude that SNL after NAC appears to lower surgical procedures and can benefit patients with downstaging of the axilla from less axillary treatment[16].

In those patients with clinically positive axilla previous to NAC, three recently published prospective studies, ACOSOG Z1071, SENTINA and SN FNAC have shown that SLN false negative rates are directly related to the technique, the number of SLNs excised and the size of the SLN metastases after NAC[17-19]. In the ACOSOG Z0071, in 525 women who met the eligibility criteria, the SLN identification rate was 92.5%. The use of dual technique (radioisotope and blue dye) and the excision of ≥ 2 SLNs lower the false negative rates to 10.8% and 12.8% respectively. Because the FN rate was higher than the pre-established 10%, additional analysis of factors that influences the FN rates should be assessed[17]. The SENTINA trial, a four arm prospective multicenter cohort study, included patients with SLN before NAC and after NAC. In 592 patients with clinically positive axillary nodes before NAC who downstaged to node negative after NAC underwent SLN biopsy plus ALND. In this group, FN rates dropped to 9.6% when ≥ 2 SLNs were removed and to 8.6% when the dual technique (blue + radioisotope) was used[18]. The third study, the SN FNAC study included 153 patients with biopsy proven positive axillary nodes before NAC. Rates of FN were 9.6% with an identification rate of 87.6%. Similarly to the other studies, when 2 or more SLNs were removed the FN dropped to 4.9% that improves significantly the FN rates compared to the previous studies. Interestingly, this study analyses the FN rates related to the inclusion or not of isolated tumor cell (ITC) in the SLN as positive or negative staging. In those patients where ypN0(i+) were considered negative nodes, the FN increased to 13.3%, indicating the importance of including any residual tumor burden in the SLN as a positive node[19].

MINIMAL SLN INVOLVEMENT IN THE SLN (ISOLATED TUMOR CELLS)

Since the introduction of the SLN, we have learnt that the more thoroughly examination of the SLNs has increased the detection of minimal metastasis in the SLNs. Traditionally, routine hematoxiline-eosine (H and E) staining has been used to identify lymph node metastasis, and with the introduction of immunohistochemistry (IHC) staining the detection of ITC has come into the scenario. Recent studies showed a 10% increased in detection of micrometastasis in the SLN when using more extensive examination[20]. The outcome of histopathological analysis has implications in the surgical and adjuvant treatment of breast cancer patients. Staging breast cancer relies heavily on the status of the lymph nodes and the 6th edition incorporated the ITCs and micrometastasis

Table 1 False negative rates in the randomized trials of sentinel lymph node after neoadjuvant chemotherapy in patients with axillary metastasis before neoadjuvant chemotherapy

	ACOSOG Z1071	SENTINA	FN SNAC
No. of patients	756	592	153
FNR with 1 SLN	31.5%	24.3%	18.2%
FNR with > 2 SLNs	12.6%	9.6%	4.9%
FNR with single tracer	20.3%	16%	16%
FNR with dual tracer	10.8%	8.6%	5.2%
FNR with N0(i+) as positive	8.7%	-	8.4%

FNR: False negative rates; SLN: Sentinel lymph node.

into their classification. As the size of SLN metastasis increases, the rate of non-SLN metastasis size also increased from around 4% in the ITC, to 5%-19% in the micrometastasis and around 50%-60% in the macrometastasis[20-22].

The prognostic implications of minimal lymph node involvement (*i.e.*, isolated tumor cells, micrometastasis) in early breast cancer have been long debated. The impact of finding this minimal metastasis in the SLN in the adjuvant setting has been reported extensively with different outcomes due to the great variability in patient population, tumor characteristics, histology assessment and so on[22,23]. Even more, the significance of micrometastasis in patients with ALND seems to be worst than in patients with SLN, making more difficult to establish its real significance[24]. It is important to consider that the studies that reported improved disease free survival in patients with SLN micrometastasis or ITCs are the ones where the majority of patients receive systemic treatments, and in this can also influence how to manage the axilla surgically[22].

To shed light to this subject, the ACOSOG Z10 trial with 5184 patients, showed that IHC detected metastasis in neither the SLN (P = 0.66) nor bone marrow (P = 0.08) were independent predictors of overall survival, although bone marrow status showed a strong trend on multivariate analysis. SLN IHC detected metastasis appear to have no impact on overall survival[25], because in the Z0010 trial treatment decisions were not based in the IHC results, the significance of ITCs may be better determined. Since the report of this trial, in many centers IHC has been abandoned for the assessment of SLN in the adjuvant setting.

Despite the knowledge of the prognosis of minimal involvement of SLNs in the adjuvant setting, this cannot be extrapolate to the neoadjuvant setting and actually, there is no such studies in the NAC setting. Rates of positivity of non sentinel nodes with a micrometastasis in the SLN in patients with NAC have been reported to be between 12% to 50%[13-15] and the SLN is the only positive node in around 50% of cases, rates lower than the adjuvant setting[15].

It is likely that micrometastasis in the SLN in patients after NAC has a different meaning than micrometastasis in the SLN in adjuvant therapy. Micrometastasis or ITC in the SLN in NAC patients could represent the presence of minimal nodal disease pretreatment which did not respond to therapy or the remnants of macroscopic nodal disease which has had a partial response to the treatment and in this way it has been addressed in the 7th edition of the AJCC[26], where ypN0(i+) is considered residual disease in the SLN. Maybe, the classification of ITC after NAC under N0 should be revised although follow up on these patients is required to assess the real prognostic value of the ITC after NAC.

The number of residual metastastic axillary nodes after NAC has been established as an important prognostic factor for disease free survival[6]. Axillary response after NAC is a better prognostic factor than response of the primary tumor[6,8,27].

Because most of these studies included patients with ALND, ITC in the axillary nodes are not reported. But one of the most important finding of the SN FNAC trial is that metastasis in the SLN after NAC of any size influences the rate of FN results, so ITC in the SLN after NAC should be considered positive[19]. In the ACOSOG Z0071, SLNs were not examined by IHC and positive SLNs were defined as those with metastasis higher than 0.2 mm, so ITC when reported were considered as node negative[17]. Data from the trial presented at the San Antonio Breast cancer Conference suggested that FN rate could be improved when ITC were included in the analysis as positive nodes, in these cases, FN rates decreased to 8.7% (Table 1). Also, our group presented data at the Society of Surgical Oncology assessing the overall survival (OS) of patients depending on the response to NAC treatment. A SLN biopsy was performed in 118 patients (32.5%). Eleven (9.3%) patients had residual ITCs in the SLN. When analyzing OS by axillary response, patients with ypN0(i+) who had a clinically negative axilla at diagnosis (cN0) had similar OS than those with pathologic complete response in the axilla, while those with ypN0(i+) who had a clinically positive axilla before NAC treatment (cN+) had a worse OS. This results suggest the importance of the ITCs in the SLN after a proven axillary metastasis before NAC, although these results need to be regarded with caution as the number of patients with ypN0(i+) were low in our study[27].

In conclusion, SLN after NAC in patients with biopsy proven positive axillary nodes before NAC is feasible and accurate when surgical technique is improved by excising 2 or more SLNs, and by using a dual technique. False negative rates can be lowered when considering ITCs as positive nodes, suggesting that any size of metastasis in the SLN after NAC is important. Further follow up on this group of patients is needed to know the prognostic implications of the ITCs in the SLN after NAC.

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EDITORIAL

Microenvironment and endocrine resistance in breast cancer: Friend or foe?

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Abstract

Breast cancer affects one in eight women around the world. Seventy five percent of these patients have tumors that are estrogen receptor positive and as a consequence receive endocrine therapy. However, about one third eventually develop resistance and cancer reappears. In the last decade our vision of cancer has evolved to consider it more of a tissue-related disease than a cell-centered one. This editorial argues that we are only starting to understand the role the tumor microenvironment plays in therapy resistance in breast cancer. The development of new therapeutic strategies that target the microenvironment will come when we clearly understand this extremely complicated scenario. As such, and as a scientific community, we have extremely challenging work ahead. We share our views regarding these matters.

Key words: Breast cancer; Tumor microenvironment; Endocrine resistance; Tamoxifen; Stroma; Estrogen receptor; Aromatase inhibitors; Cancer stem cells

Core tip: Resistance to endocrine therapy in breast cancer is an important clinical problem that requires further insight to develop a solution. We here discuss a paradigm shift, where the interplay of the tumor cells with the microenvironment, and the role of cancer stem cells are discussed as key targets in the development of novel therapeutic strategies.

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WHY DOES ENDOCRINE RESISTANCE DEVELOP?

Breast cancer is the most frequent cancer in women in the Western world and one of the main causes of death. Seventy five percent of breast cancer patients have estrogen receptor-alpha (ERα) positive tumors and endocrine therapy is the adjuvant treatment of choice in this scenario. However, a high percentage of patients develop resistance and cancer reappears; up to one third of patients recur within 15 years of the initial diagnosis[1]. Resistance to endocrine therapy is considered as *de novo* when there is no primary response to treatment. However, when therapy is initially successful but cancer eventually recurs, endocrine resistance is considered as acquired[2].

The most widely used endocrine therapy for breast cancer patients has been tamoxifen, a selective ER modulator. Tamoxifen was developed in the 70's and is considered the first targeted therapy for cancer, as it specifically targets the ER[3]. Other endocrine treatments include selective ER down modulators such as Fulvestrant and aromatase inhibitors like Letrozole and Anastrozole[4].

A number of mechanisms have been proposed as responsible for inducing acquired tamoxifen resistance. In particular a great number of papers have historically dealt with alterations in growth factor receptor pathways, in particular the HER family of growth factor receptors, as the responsible for this phenomenon[5]. A quick search in PubMed while we are writing this editorial shows that when we use the key words "breast cancer and tamoxifen resistance" we find 1869 publications; if the word "growth factor" is added, the number is reduced to 503 and "Her-2" leads to 236. However, if we look into other plausible mechanisms very few papers are found: For example adding the word "microenvironment" leads to 17 citations, "inflammation" accounts for 4, "integrins" 4, "stroma" 15, "fibroblasts" 40 and "stem cells" leads to 40. However, when we look at the first publications related to these topics we find that the first paper listed in PubMed related to growth factors and tamoxifen resistance was published in 1988[6], whereas "stem cells", for example, dates to 1985[7]. So evidently researchers have been thinking about other mechanisms but probably the means to carry out these investigations were not available, or very few researchers thought that mechanisms other than autocrine loops within the tumor cell population could be responsible for the progression of the disease. We know today, however, that tumors are not only composed of neoplastic cells themselves, but that other cell types and extracellular components are critical both to tumor progression and response to treatment[8]. Thus, considering these as key players in the development of endocrine resistance is critical. The following paragraphs aim at highlighting some of the main findings related to endocrine resistance through mechanisms that need extensive research to lead us to the development of novel strategies for the treatment of breast cancer.

INFLAMMATION AND ENDOCRINE RESISTANCE

A growing body of evidence supports the role of the immune system as a regulator of tumor development and dissemination. Infiltrating immune cells produce cytokines, proteinases, chemokines and growth factors that promote extracellular matrix remodeling and angiogenesis. In particular gene-profiling studies have linked inflammation related gene clusters to resistance in patients treated with tamoxifen[9] and the aromatase inhibitor Anastrozole[10]. Moreover, a number of cytokines have been associated to suppression of ERα in breast cancer cells such as TNF, IL1β, IL6 and amphiregulin[11]; ER negative tumors are associated to more aggressive and invasive phenotypes. Epithelial to mesenchymal transition can be induced by factors such as IL6 with the upregulation of stem-related transcription factors[12,13]. Moreover, increased IL6 serum levels are correlated with decreased response to endocrine therapy in breast cancer and poorer survival[14,15]. In the ERα negative scenario IL1β is correlated with increased invasiveness and poor prognosis[16]. In ERα expressing breast tumors, IL1β has been shown to activate ER's transcriptional activity[17,18] and to modulate the response to 4-OH-tamoxifen; in particular in the presence of IL1β tamoxifen acts as an agonist instead of an antagonist[19]. The SDF-1-CXCR4 axis has also been implicated in breast tumor progression[20,21]. CXCR4 overexpression is correlated with worse prognosis and decreased survival in both the ER positive and negative scenario[21,22]. Moreover, using MCF-7 cells, treatment with SDF-1 induced tamoxifen and fulvestrant resistance in cells overexpressing CXCR4[21].

Tumor associated macrophages (TAMs) are associated to increased angiogenesis and survival[23]. Results from experimental models in mice suggest that TAMs are key players in the progression to metastasis[24]. Moreover, experiments suggest that TAMs produce estrogens that directly stimulate the proliferation of ERα positive breast cancer cells[25]. CD68, a macrophage marker, has been associated to increased recurrence suggesting that in ER positive breast cancers the presence of macrophages may lead to endocrine resistance[26].

STEM CELLS AND ENDOCRINE RESISTANCE

Cancer stem cells have gained attention in the last years as responsible for tumor progression and resistance to therapy[27]. Experiments carried out using human samples have clearly shown that both chemo

and radiotherapy increase the percentage of breast cancer stem cells in a neo-adjuvant setting[28]. In the context of endocrine therapy our results together with those of other groups strongly suggest that endocrine treatment leads to enrichment in breast cancer stem cells. Our working hypothesis, based on the literature and our results, is that breast cancer stem cells express reduced levels or no ER-α, and would thus not be efficiently targeted by endocrine treatment[29]. A study testing the effect of neo-adjuvant treatment in patients with Letrozole shows that it leads to enrichment in cells with mammosphere forming capacity[30]. Simões *et al*[31] analyzed the impact of estrogen signaling on MCF-7 mammosphere forming capacity. They plated MCF-7 cells straight onto nonadherent plates and treated the suspension cultures with hormones finding that estradiol decreased, and 4-OH-tamoxifen increased mammosphere forming capacity. The same results were true for suspension cultures of primary human normal and tumor breast cell suspensions, where treatment with 4-OH-tamoxifen led to an increase in Nanog and Sox-2. Ao *et al*[32] on the other hand treated suspension cultures with 4-OH-tamoxifen and then passaged the cells to media without antiestrogen (still in suspension) and found that under these conditions a greater amount of mammospheres were formed. We showed that tamoxifen selects for cells with stem cell properties in the human MCF-7 cells line, as well as in mouse LM05-E cells and the M05 tumor from which they derive[33]. Mammosphere assays revealed that pretreatment of either cell line with 4-OH-tamoxifen leads to an increase in cells with increased clonogenicity in suspension. Additionally, we analyzed the gene expression of transcription factors associated to pluripotency and found that they were increased both in the mammospheres and in cells growing on 2D treated with 4-OH-tamoxifen for 5 d. *In vivo* studies using the M05 tumor showed similar results with an increase in the amount of cells with mammosphere forming capacity in tumors derived from mice treated with tamoxifen containing pellets. These tumors were enriched in $CD29^{h}/CD24^{l}$ cells, in comparison to the parental tumor. Additionally, when passaged to untreated mice, those tumors that derived from mice that had been previously exposed to tamoxifen generated "secondary tumors" that grew at a faster rate compared to controls, and had a higher capacity of giving rise to mammospheres as well as maintaining an increased $CD29^{h}/CD24^{l}$ cell population. Finally, M05 tumor passages that had progressed to hormone independence had a higher amount of cells with mammosphere forming capacity supporting the notion that increased aggressiveness and endocrine independence are correlated with an increase in cells with stem cell properties[33]. These results, in conjunction, strongly suggest that breast cancer stem cells are involved in endocrine resistance.

THE EXTRACELLULAR MATRIX, CANCER ASSOCIATED FIBROBLASTS AND ENDOCRINE RESISTANCE

Cancer associated fibroblasts have long been believed to play a key role in cancer progression[34]. In breast cancer in particular, several lines of evidence strongly suggest that they are vital in determining tumor progression and the outcome of therapy[35]. A seminal paper by Finak *et al*[36] identified distinct stromal signatures that corresponded to good and poor-outcome breast cancers. They were able to identify a 26 gene predictor that forecasted disease outcome with higher precision than predictors or signatures derived from whole tissues. In this line of evidence, but in the context of endocrine resistance, an extracellular matrix gene cluster has been associated to prognosis and response to treatment[37]. In particular they found that fibronectin, lysyl oxidase, SPARC and TIMP3 expression levels were associated to the prognosis of patients with breast cancer whereas levels of tenascin C were associated to resistance to treatment with tamoxifen. A recent paper by Holton *et al*[38] using 3D cultures and Fourier transform infrared spectroscopic imaging shows that fibroblasts induce epithelial to mesenchymal transition in cancer cells together with a downregulation in ERα levels. Our work also suggests that stromal factors modulate response to endocrine resistance. In particular we showed that conditioned media derived from carcinoma associated fibroblasts induced tamoxifen resistance in otherwise sensitive cells, using a mouse model of estrogen dependent breast cancer[39,40]. Moreover, we found that fibronectin, which is mostly produced by fibroblasts in breast tumors, induces resistance in both LM05-E and MCF-7 cells. This effect is accompanied by an induction in ERα phosphorylation at serine-118. Interestingly, high levels of phospho-serine-118 has been previously associated to endocrine resistance in breast cancer[41].

BACK TO THE BEGINNING

The examples above are just a snapshot of the recent findings regarding endocrine resistance and microenvironment. So is the microenvironment a friend or a foe in this context? Clearly I believe that we are just beginning to unravel the complexity of cancer and that evidently there is no single culprit to failure when it comes to treatment. However, we definitely need a lot of work to be carried out to understand exactly what the role of each player is in this complicated challenge. Moreover, the work that needs to be done is extremely delicate given that it implies analyzing and understanding how tumor and tumor "associated" cells behave in different contexts. This type of work is time consuming, needs to be carried out in different model systems and is very expensive. The challenge I believe

is patience. The other key point here is that researchers that study different types of cancer need to interact more and companies must be willing to openly share new chemicals when requested even though they are not being developed for the purpose the scientist asking is wanting to explore.

The road ahead is exciting and may result frustrating at times. We are just beginning to understand that the microenvironment plays a key role in endocrine resistance in breast cancer. The numbers in PubMed are clear evidence that we are in the dawn of our understanding in this matter. As the poet Robert Frost would say, we are taking the road less travelled, and that should make the difference.

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EDITORIAL

Combination therapies improve the anticancer activities of retinoids in neuroblastoma

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Abstract

Most therapeutic protocols for child cancers use cytotoxic agents which have a narrow therapeutic index, and resulting in severe acute and chronic toxicities to normal tissues. Despite the fact that most child cancer patients achieve complete remission after chemotherapy, death still occurs due to relapse of persistent minimal residual disease (MRD) which remaining after initial cytotoxic chemotherapy. Advanced neuroblastoma (NB) is a leading cause of cancer deaths in young children. Retinoids are an important component of advanced NB therapy at the stage of MRD, yet half of all patients treated with 13-*cis*-retinoic acid still relapse and die. More effective combination therapies, with a lower side-effect profile, are required to improve outcomes for NB. Fenretinide or N-4-hydroxyphenyl retinamide is a synthetic derivative of retinoic acid which works on cancer cells through nuclear receptor-dependent and -independent signalling mechanisms. Moreover, several histone deacetylase inhibitors have entered early phase trials, and, suberoylanilide hydroxamic acid has been approved for use in adult cutaneous T cell lymphoma. A number of studies suggest that retinoid signal activation is necessary for histone deacetylase inhibitor activity. A better understanding of their mechanism of actions will lead to more evidence-based retinoid combination therapies.

Key words: Retinoids; Histone deacetylase inhibitors; Combination therapies; Neuroblastoma; Fenretinide

Core tip: Neuroblastoma (NB) begins in embryonal neural crest cells, which later give rise to the sympathetic nervous system, and is caused in part by factors which arrest differentiation. *In vitro*, retinoids force susceptible cancer cells down a pathway of terminal differentiation and, have been part of the routine treatment of advanced NB for number of decades. Synergistic anti-tumour activity between histone deacetylase inhibitors and retinoids has been observed in a variety of preclinical models. This editorial note discusses some of these findings on the combination therapies for improving the anticancer activities of

retinoids in NB.

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INTRODUCTION

Neuroblastoma (NB) is a tumor of the sympathetic nervous system and the most common extracranial solid tumour in childhood[1]. NB accounts for more than 7% of malignancies in patients younger than 15 years and around 15% of all paediatric oncology deaths[2]. Some infants experience spontaneous regression, whereas older patients have maturation of their tumor into benign ganglioneuromas. However, the outcome for children with a high-risk clinical phenotype has improved only modestly, with long-term survival still less than 40%[3]. The introduction of 13-*cis*-retinoic acid (13-*cis*-RA) in the therapy of NB has improved the prognosis of this disease. Currently, the standard treatment for high risk of NB consists of myeloablative therapy followed by autologous hematopoietic stem cell transplantation and maintenance with 13-*cis*-RA for the treatment of minimal residual disease (MRD), leading to a 3-year disease-free survival rate of about 50%[4]. Retinoids are an important component of advanced NB therapy at the stage of MRD, yet half of all patients treated with 13-*cis*-retinoic acid still relapse.

Retinoid therapy in paediatric cancer

Retinoids are vital for the growth and differentiation of a variety of normal adult and embryonic tissues, and have potent antiproliferative effects on many malignant cell types[5]. Retinoids mediate their widespread effects on cells by regulating the transcription of target genes through a complex system of ligand-inducible nuclear transcription factors: The retinoic acid receptors and retinoid X receptors[6]. RA exists in several stereoisomeric forms: Predominantly *all-trans* retinoic acid (ATRA) and 13-*cis*-RA, but also as less-stable isomers such as 9-*cis* retinoic acid. In the last few decades they have been widely studied in cancer prevention and therapy because of their ability to induce differentiation of tumor cells[7]. Retinoids are successfully used for the treatment of one pediatric cancer: Acute promyelocytic leukemia. ATRA converts the PML-RAR-α fusion protein into activator of transcription and restores cell differentiation[8]. Retinoids have also been widely investigated in solid tumors, especially in NB. In a long-term study for children with high-risk NB treated on a randomized trial of myeloablative therapy followed by 13-*cis*-RA, which given after intensive therapy resulted in significant improvement in 5-year overall survival rates, regardless of the type of consolidation[9]. However, as many high-risk patients still ultimately die due to relapse of persistent MRD after initial cytotoxic chemotherapy, novel therapies effective against MDR NB are needed.

Fenretinide is an effective retinoid therapy

Retinoids are vitamin A analogues required for normal morphogenesis and maintenance of diverse embryologic and adult tissues, which act on cells by binding nuclear receptors[10]. Fenretinide or N-4-hydroxyphenyl retinamide (4-HPR) is a synthetic derivative of retinoic acid which works on cancer cells through nuclear receptor-dependent and -independent signalling mechanisms[11]. 4-HPR has a broad spectrum of cytotoxic activity against primary tumor cells, cell lines, and/or xenografts of various cancers, including NB [11-13] and has been tested in early phase clinical trials in recurrent NB [14,15]. 4-HPR was anti-angiogenic in multiple tumour types and cytopathic in some cancer cells which were resistant to other retinoids or chemotherapeutics[13]. Clinical trials have revealed that 4-HPR is a highly active therapeutic and chemo-preventive agent with minimal side-effects in NB[14]. A phase Ⅰ/Ⅱ trial of oral 4-HPR in children with high-risk, relapsed solid tumours demonstrated minimal 4-HPR toxicity, but only stable disease as the best clinical response[16].

Combination therapy improves the anticancer activity of histone deacetylase inhibitors and retinoids

Increased histone deacetylase activity is a common causal factor in human cancer that causes transcriptional silencing of tumour suppressor genes[17]. Histone deacetylase inhibitors prevent deacetylases removing acetyl groups from histone tails, thereby promoting gene transcription[18]. Several histone deacetylase inhibitors have entered early phase trials, and, suberoylanilide hydroxamic acid (SAHA) has been approved for use in adult cutaneous T cell lymphoma[19]. The histone deacetylase inhibitor side-effect profile is low when compared with cytotoxic chemotherapy[20]. Moreover, two unbiased preclinical screens identified retinoid signal activation as the most effective method of augmenting the histone deacetylase inhibitor anti-cancer signal[21,22]. Retinoic acid receptor α (RARα), and, preferentially expressed antigen of melanoma, both repressor proteins for the retinoid signal, was shown to mediate resistance to histone deacetylase inhibitors[21]. Furthermore, RARα-deficient cells showed enhanced sensitivity to histone deacetylase inhibitors *in vitro* and *in vivo*[22]. These studies suggest that retinoid signal activation is necessary for histone deacetylase inhibitor activity. Hahn *et al*[23] used an HDAC inhibitor (valproicacid) as an enhancer to screen a small-molecule library for compounds inducing NB maturation, the top hit identified in the screen was all-trans-retinoic acid. These studies demonstrated that investigation of HDAC inhibitors and retinoids in combination are warranted to improve the anticancer activities in cancer.

Combination therapies improve the anticancer activities of retinoids in NB

Synergistic anti-tumour activity between histone deacetylase inhibitors and retinoids has been observed in a variety of preclinical models[24,25]. A study suggested that the HDAC inhibitor LAQ824 has a greater antitumor activity in combination with 13-*cis*-retinoic acid in melanoma tumors[24]. Another study showed that the intracranial tumors in ND2:SmoA1 mice treated with retinoid acid + SAHA + cisplatin showed a 4-fold increase in apoptosis over controls, and a 2-fold increase over animals receiving only SAHA or retinoid acid + SAHA[25]. We and others have shown that retinoids combined with histone deacetylase inhibitors are synergistic[26,27]. However, SAHA combined with 13-*cis*-retinoic acid, was well-tolerated in a phase Ⅰ/Ⅱ paediatric trial, but the best response for relapsed solid tumour patients was stable disease[28]. Recently, our study showed that 4-HPR+SAHA as a more effective therapy for NB than 13-*cis*-RA alone or with SAHA[29]. The 4-HPR + SAHA combination induced caspase-dependent apoptosis through activation of caspase 3, reduced colony formation and cell migration *in vitro*, and tumorigenicity *in vivo*. The 4-HPR and SAHA combination significantly increased mRNA expression of thymosin-beta-4 (Tβ4) and decreased mRNA expression of RARα. Importantly, the up-regulation of Tβ4 and down-regulation of RARα were both necessary for the 4-HPR + SAHA cytotoxic effect on NB cells. Moreover, Tβ4 knockdown in NB cells increased cell migration and blocked the effect of 4-HPR + SAHA on cell migration and focal adhesion formation[29]. This study demonstrates that Tβ4 is a novel therapeutic target in NB, and that 4-HPR and SAHA is a potential combination therapy for the disease.

CONCLUSION

A therapeutic role for retinoids and HDAC inhibitors in several human cancer types, including NB, is well established. However, retinoids and HDAC inhibitors are not completely effective anti-cancer agents when used alone; thus, a better understanding of their mechanism of actions will lead to more evidence-based retinoid combination therapies. Because differentiation is aberrant in NB, compounds that modulate transcription and induce differentiation, such as HDAC inhibitors and retinoids, are of particular interest. Further studies to understand the mechanism of drug actions and the clinical trials with large cohort of patients to determine the efficacy of HDAC inhibitors and retinoids for patients with high-risk NB are warranted.

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EDITORIAL

Direct therapeutic intervention for advanced pancreatic cancer

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Abstract

Currently, chemotherapy is an accredited, standard treatment for unresectable, advanced pancreatic cancer (PC). However, it has been still showed treatment-resistance and followed dismal prognosis in many cases. Therefore, some sort of new, additional treatments are needed for the better therapeutic results for advanced PC. According to the previous reports, it is obvious that interventional endoscopic ultrasonography (EUS) is a well-established, helpful and low-risky procedure in general. As the additional treatments of the conventional therapy for advanced PC, many therapeutic strategies, such as immunotherapies, molecular biological therapies, physiochemical therapies, radioactive therapies, using siRNA, using autophagy have been developing in recent years. Moreover, the efficacy of the other potential therapeutic targets for PC using EUS-fine needle injection, for example, intra-tumoral chemotherapeutic agents (paclitaxel, irinotecan), several ablative energies (radiofrequency ablation and cryothermal treatment, neodymium-doped yttrium aluminum garnet laser, high-intensity focused ultrasound), *etc.*, has already been showed in animal models. Delivering these promising treatments reliably inside tumor, interventional EUS may probably be indispensable existence for the treatment of locally advanced PC in near future.

Key words: Interventional endoscopic ultrasonography; Advanced pancreatic cancer; Radiofrequency ablation; Gemcitabine; Endoscopic ultrasonography guided-fine needle injection; Dendritic cells

Core tip: Unresectable, advanced pancreatic cancer (PC) has been still showed treatment-resistance and followed dismal prognosis in many cases with conventional therapies. Therefore, some sort of new, additional treatments are needed for the better therapeutic results for advanced PC. In recent years, interventional endoscopic ultrasonography (EUS) has been developed, disseminated and used efficiently all over the world as indispensable therapeutic strategies for PC. Therapeutic trials by interventional EUS for advanced PC until now, and describe the possibilities and expectations of anti-

tumor therapy for advanced PC by interventional EUS to the future through this epoch-making deployment are summarized in this Editorial.

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TEXT

In recent years, interventional endoscopic ultrasonography (EUS) has been developed, disseminated and used efficiently all over the world as indispensable therapeutic strategies for various diseases of digestive area, such as malignant tumors, drainage, pain relief and recurrent lesions. Besides, unresectable, advanced pancreatic cancer (PC) has been still showed treatment-resistance and followed dismal prognosis in many cases. Some sort of new, additional treatments are needed for the better therapeutic results. Because of the merit which approaches inside the pancreas directly through stomach or duodenum, interventional EUS may be a potential target of crucial treatment strategy. Different strategies of interventional EUS for advanced PC have been conducted, and will also be carried out in the future. We hope to summarize the therapeutic trials by interventional EUS for advanced PC until now, and describe the possibilities and expectations of anti-tumor therapy for advanced PC by interventional EUS to the future through this epoch-making deployment in this Editorial.

Since EUS techniques allow access to pancreas in a comparatively minimally invasive fashion, it is a feasible procedure for the potential of a targeted delivery of therapeutic agents for PC by fine needle injection (FNI) through gastric or duodenal wall. Hence, many therapeutic trials for advanced PC by EUS guided-FNI (EUS-FNI) with a curative intent have been conducted so far. EUS-FNI involves direct intra-tumoral delivery of anti-tumor agents under EUS guidance for local control of tumor growth in patients with unresectable PC. As opposed to systemic administration, direct treatment is able to effect the targeted lesion of cancer without many normal lesions. Therefore, the EUS-FNI technique offers theoretic potential to deliver high dose concentration while minimizing systemic side effects. In addition, immune-modulating cells such as mixed lymphocyte and dendritic cells (DCs) can also be injected into PC as a potential anti-tumor therapy. However these results were not fulfilled the expected level as well as conventional treatments for advanced PC.

Chang *et al*[1] RFA conducted a phase Ⅰ trial in which 8 patients with advanced PC were given intra-tumoral injections of activated allogenic mixed lymphocyte culture (cytoimplant) guided by EUS. In this report, no patient had treatment-related pancreatitis in the procedures. However, the trial was suspended and final results have not been published. Irisawa *et al*[2] reported a pilot study about EUS-FNI of immature DCs into advanced PC. In 7 patients with unresectable PC who previously failed a chemotherapeutic agent, gemcitabine. DCs are potent antigen-presenting cells which have ability to initiate $CD4^+$ helper and $CD8^+$ cytotoxic T lymphocytes (CTLs)-mediated anti-tumor immune responses[3]. In the report, injected immature DCs may intake apoptotic/necrotic pancreatic tumor cells and present tumor-associated antigenic peptides into MHC class Ⅰ and Ⅱ molecules on DCs, resulted in induction of antigen-specific CTLs. There were 3 partial responses (PR), 2 patients with stable disease (SD). Median survival was 9.9 mo without complication associated with EUS-FNI procedure. The results have not been achieved satisfactory level, however it is hopeful to publish the final results about the project. Apart from that, a combination therapy of chemotherapy (gemcitabine) with immunotherapy (OK-432-stimulated mature DCs) using EUS-FNI, followed by intravenous infusion of lymphokine-activated killer cells stimulated with anti-CD3 monoclonal antibody has reported[4]. In this report, 5 patients with inoperable locally advanced PC had been treated. No serious treatment-related adverse events were observed during the study period. One patient had PR and 2 had long-SD more than 6 mo in this regimen. Demonstrating in many more number of patients with locally advanced PC will be desired.

In locally tumors, induction of tumor necrosis factor-alpha (TNF-α), which is a pro-inflammatory cytokine can induce tumor necrosis and shrinkage. A phase Ⅰ clinical trial using TNFerade *via* EUS-FNI in combination with radiation for patients with advanced PC therapy has been reported[5]. TNFerade is a replication-deficient adenovirus vector carrying the human *TNF-α* gene regulated by a radiation-inducible promoter (Egr-1). Intra-tumoral TNFerade with radiation has been shown to be safe in a phase Ⅰ clinical trial of 30 patients with PC[5]. In addition, a phase Ⅰ/Ⅱ trial was conducted for 50 patients with advanced PC, using TNFerade in combination with chemoradiation therapy. In the study, TNFerade was delivered by EUS guidance for 27 patients without severe procedure-related complications[6]. Over a 5-wk treatment period, 1 patient had complete response, 3 had PR, and 12 patients had SD. The results showed a trend toward improved overall survival, however, it was not statistically significant. Moreover, the strategy is only suitable for patients with locally advanced PC. Although the clinical results suggest that TNF-α may be a useful candidate for locally advanced PC therapy, clinical benefits remain unknown. Subsequently, ONYX-015, an oncolytic attenuated adenovirus that preferentially replicates in malignant cells, leading to cell death had been introduced into PC[7]. Hecht *et al*[8] completed a phase Ⅰ/Ⅱ trial of EUS FNI-guided intra-tumoral delivery of ONYX-015 combined with gemcitabine in 21 patients with advanced PC. Four patients developed comparatively severe complications, such as sepsis

and duodenal perforations which were attributed to the EUS procedures, in spite of no convincing efficacy of ONYX-015 was found. The median survival was 7.5 mo that has no significant difference with the conventional therapies.

Otherwise, EUS injectable anti-tumor agents, there are EUS-guided coagurative therapies. Radiofrequency ablation (RFA) therapy guided by EUS for advanced PC has not been actually clinical trial, because of the poorly accessible PC, in spite of the feasibility and effectiveness was confirmed in a porcine model[9]. Indeed, RFA provides localized tissue ablation within 1 cm zone from the FNI needle catheter. Another ablative technique is photodynamic therapy (PDT), which is more selective than RFA. The safety and efficacy of PDT guided by EUS for advanced PC was also demonstrated in a porcine model[10]. EUS-guided low-dose PDT may be safe and feasible for advanced PC, without no significant procedure-related complications. Moreover, brachytherapy using iodine-125 or palladium-103 has been successfully placed directly into tumors for the treatment of patients with PC. Pilot studies by Sun *et al*[11] in 15 patients and by Jin *et al*[12] in 25 patients with unresectable PC showed the safety and feasibilty of EUS-guided brachytherapy. However, it may be needed to solve the mechanical difficulties of inserting solid seeds for contributing and disseminating worldwide.

Conceivable causes of the limited therapeutic effects by EUS-FNI for advanced PC are wide varieties. Primarily, advanced PC has extremely aggressive nature originally and increases momentarily. As the other conventional treatments, it is uneasy to overwhelm the progression of advanced PC. Secondly, many factors, such as genetic alterations, cellular dynamics and influences of intracellular or microenvironmental stress are intricately entangled in the development of PC. In existing state, it has never demonstrated the clinical effect by one kind of drug or tool alone for advanced PC. Thirdly, advanced PC has a feature of stubborn object because of the high density of fibrosis due to intense parenchymal inflammation. So that, it is incapable of piercing into PC without difficulty and penetrating injected solution adequately inside tumor in many cases. Lastly, even if the efficacy of injected solution is crucial, it is briefly uncontrollable the metastasis, invasion and angiogenesis of the PC, because EUS-FNI is only regional treatment. It may probably be needed to combine with some other systematic treatments for advanced PC in actual clinical application.

Currently, chemotherapy is an accredited, standard treatment for unresectable, advanced PC. According to the previous reports, it is obvious that interventional EUS is a well-established, helpful and low-risky procedure in general. The problems of interventional EUS as the decisive treatments for advanced PC that we must overcome are not the endoscopic procedures but how the therapeutic agents are delivered accurately and what are inserted directly inside the tumor. As the additional treatments of the conventional therapy for advanced PC, many therapeutic strategies, such as immunotherapies, molecular biological therapies, physiochemical therapies, radioactive therapies, using siRNA, using autophagy have been developing in recent years. Moreover, the efficacy of the other potential therapeutic targets for PC using EUS-FNI, for example, intra-tumoral chemotherapeutic agents (paclitaxel, irinotecan), several ablative energies (RFA and cryothermal treatment, neodymium-doped yttrium aluminum garnet laser, high-intensity focused ultrasound), *etc.,* has already been showed in animal models[13-17].

In conclusion, delivering these promising treatments reliably inside tumor, interventional EUS may probably be indispensable existence for the treatment of locally advanced PC in near future.

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EDITORIAL

Tumor biology in estrogen receptor-positive, human epidermal growth factor receptor type 2-negative breast cancer: Mind the menopausal status

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Abstract

Breast cancer is not one disease, but can be categorized into four major molecular subtypes according to hormone receptor [estrogen receptor (ER) and progesterone receptor (PgR)] and human epidermal growth factor receptor type 2 (HER2) expression status. Ki67 labeling index and/or multigene assays are used to classify ER-positive, HER2-negative breast cancer into luminal A and luminal B (HER2-negative) subtypes. To date, most studies analyzing predictive or prognostic factors in ER-positive breast cancer have been performed in postmenopausal women, mainly using patients and samples in adjuvant aromatase inhibitor trials. In contrast, even the clinical roles of PgR and Ki67 have been little analyzed so far in premenopausal women. PgR is one of the estrogen-responsive genes, and it has been reported that plasma estradiol levels are related to expression levels of estrogen-responsive genes including *PGR* in ER-positive breast cancer. In this article, biological differences, especially differences in expression of PgR and Ki67 in ER-positive breast cancer between pre- and postmenopausal women are discussed. Clinical roles of PgR and Ki67 in ER-positive breast cancer differ between pre- and postmenopausal women. We suggest that the mechanisms of development and estrogen-dependent growth of ER-positive breast cancer might differ according to menopausal status.

Key words: Breast cancer; Progesterone receptor; Estrogen receptor; Ki67; Menopausal status

Core tip: Progesterone receptor (PgR) is one of the estrogen-responsive genes, and it has been reported that plasma estradiol levels are related to expression levels of estrogen-responsive genes including *PGR* in estrogen receptor (ER)-positive breast cancer. In this article, biological differences, especially differences in expression of PgR and Ki67 in ER-positive breast cancer between pre- and postmenopausal women are discussed. Clinical roles of PgR and Ki67 in ER-positive breast cancer differ between pre- and postmenopausal

women. We suggest that the mechanisms of development and estrogen-dependent growth of ER-positive breast cancer might differ according to menopausal status.

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INTRODUCTION

Breast cancer is not one disease, but a group of diseases that can be categorized into four major molecular subtypes according to their expression of hormone receptors (HR) [estrogen receptor (ER) and progesterone receptor (PgR)] and human epidermal growth factor receptor type 2 (HER2). Thus they are classified as: HR+/HER2-, HR+/HER2+, HR-/HER2+, and triple negative (HR-/HER2-). Treatments need to be tailored to a patient's particular subtype, so that endocrine therapies for HR-positive breast cancer and anti-HER2 therapies for HER2-positive breast cancer are recommended as first choice regardless of whether the disease is in the early stages or has become metastatic.

Expression of ER, PgR, HER2 and the proliferation marker Ki67 in breast cancer tissues is routinely assessed by immunohistochemistry, and multigene assays have recently been introduced for estimating prognosis and treatment efficacy[1]. The choice of appropriate drug therapies, especially the indication of adjuvant chemotherapy for ER-positive, HER2-negative early breast cancer, the subtype which is diagnosed in almost 80% of breast cancer cases, is sometimes controversial. Ki67 labeling index and/or multigene assays, such as 21-gene recurrence score (Oncotype Dx), 70-gene signature (Manmaprint) and PAM50 risk of recurrence score, that classify ER-positive, HER2-negative breast cancer into luminal A and luminal B (HER2-negative) subtypes are commonly used in practice, and adjuvant chemotherapy in addition to endocrine therapy is recommended for luminal B subtype[2].

To date, most studies analyzing predictive or prognostic factors in ER-positive breast cancer have been performed in postmenopausal women, mainly using patients and samples in adjuvant aromatase inhibitor trials[3-5]. In contrast, even the clinical roles of PgR and Ki67 have been little analyzed so far in premenopausal women.

PgR is one of the estrogen-responsive genes, and it has been reported that plasma estradiol levels are related to expression levels of estrogen-responsive genes including *PGR* in ER-positive breast cancer in both pre- and postmenopausal women[6,7]. We previously investigated the expression of estrogen-responsive genes (PgR and TFF1), a progesterone-responsive gene (RANKL), ER-related genes and Ki67 in ER-positive, HER2-negative breast cancer samples, and compared the correlations between expression levels of these molecular markers and clinicopathological factors, including prognosis, between pre- and postmenopausal women. Our results suggested that the mechanisms of development and estrogen-dependent growth of ER-positive breast cancer might differ according to menopausal status[8]. Thus, host factors, such as serum levels of estrogen and progesterone might affect the expression of multiple genes in ER-positive breast cancer tissues.

In this article, biological differences, especially in PgR expression and Ki67 labeling index in ER-positive, HER2-negative breast cancer between pre- and postmenopausal women are discussed.

PgR expression in ER-positive breast cancer tissues correlates with serum estrogen levels

PgR is an estrogen-responsive gene, and its expression, together with that of ER, is routinely examined in breast cancer tissues. We previously reported that expression levels of PgR in pretreatment biopsies were not predictive of the response to the neoadjuvant aromatase inhibitor exemestane, and that expression levels of PgR were decreased in posttreatment tumors compared to their levels in pretreatment specimens regardless of the treatment response[9]. It is clear that PgR expression does not fully reflect estrogen dependence: Many PgR-negative tumors respond to tamoxifen or aromatase inhibitors[9-12]. Furthermore, it has been reported that plasma estradiol levels are related to expression levels of estrogen-responsive genes, such as *PGR* and trefoil factor 1 (*TFF1*)/*pS2*, in ER-positive breast cancer in both pre- and postmenopausal women[6,7]. Dunbier *et al*[7] examined mRNA expression of estrogen-responsive genes including *PgR* in pretreatment tumor biopsies from postmenopausal patients with ER-positive breast cancer treated with the neoadjuvant anastrozole, and pretreatment plasma estradiol levels were determined by highly sensitive radioimmunoassay. They demonstrated that plasma estradiol levels were significantly associated with expression of estrogen-responsive genes in ER-positive breast cancer.

In premenopausal women, Haynes *et al*[6,13] reported significant differences in the expression of estrogen-related genes including PgR in ER-positive breast tumors across the menstrual cycle: Gene expression of estrogen-related genes was higher when serum estradiol levels were high. They also demonstrated that expression of the progesterone-regulated gene *RANKL* was almost three-fold higher when serum progesterone levels were at their highest point of the menstrual cycle.

The study of neoadjuvant endocrine therapy in premenopausal women with ER-positive breast cancer showed that positive PgR expression status by immunohistochemistry dramatically decreased in post-treatment specimens (34.4%) compared to the values

in pretreatment biopsies (98.9%) in patients treated with neoadjuvant anastrozole plus the LHRH agonist goserelin for 24 wk, whereas the percentage of patients with positive PgR status did not change significantly from baseline (91.9%) to 24 wk (89.5%) in patients treated with neoadjuvant tamoxifen plus goserelin[14].

Taken together, these data suggest that expression levels of PgR in ER-positive breast cancer tissues are associated with serum estrogen levels in both pre- and postmenopausal women.

Biological differences between pre- and postmenopausal women with ER-positive, HER2-negative breast cancer – PgR

A study analyzing clinicopathological characteristics of breast cancer in patients registered to the Japanese Breast Cancer Registry in 2011 showed that the ER-positive rate was approximately 90% in patients in their 40s and approximately 80% in those over 50 years old, while the PgR-positive rate was approximately 85% in patients in their 40s but less than 70% in those over 50 years old[15]. We previously showed that the incidence of ER-positive, PgR-negative breast cancer in women aged 50 years or younger and in those older than 50 years were 6% and 15%, respectively, whereas for ER-positive, PgR-positive tumors, incidences were 81% and 64%, respectively[16]. Moreover, most tumors had high PgR expression in women aged 50 or younger or in premenopausal women, while the distribution of PgR expression levels was evenly spread in tumors in women over 50 years of age or in postmenopausal women[16]. This suggests that reduced circulating estrogens after menopause could be the cause in the incidence of ER-positive/PgR-negative or ER-positive/low-PgR tumors in postmenopausal women[17].

PgR expression has been reported to be a prognostic factor for postmenopausal ER-positive breast cancer patients in adjuvant aromatase inhibitor trials[3-5]. Our retrospective studies also demonstrated that high expression of PgR significantly correlated with improved disease-free survival in postmenopausal women with ER-positive, HER2-negative breast cancer[8]. In contrast, in premenopausal women, PgR expression was not associated with disease-free survival[8].

Biological differences between pre- and postmenopausal women with ER-positive, HER2-negative breast cancer – Ki67

Ki67 is a nuclear protein that is expressed during all phases of the cell cycle except the G0 phase, and is a marker of tumor proliferation[18]. Recent studies have shown that the so called "luminal A" subtype-characterized by low histological grade, low proliferation as measured by Ki67, high hormone receptor status, and negative HER2 status - is less responsive to chemotherapy, and that no preferable chemotherapy regimen could be defined for treatment of this subtype[2].

The prognostic significance of Ki67 was examined in postmenopausal women who were treated with letroszole or tamoxifen in the BIG1-98 trial[19]. It was reported that higher values (> 11%) of Ki67 labeling index were associated with worse disease-free survival. Our previous study showed that when the cutoff point for determining the division between low and high Ki67 labeling index was set at 14%, low Ki67 labeling index was strongly associated with increased disease-free survival in postmenopausal women with ER-positive breast cancer[8]. We also indicated that high expression of Ki67 (≥ 14%) was significantly associated with decreased disease-free survival in postmenopausal patients treated with adjuvant aromatase inhibitors[20]. In contrast, the best cutoff points of Ki67 labeling index for disease-free survival were 30% for premenopausal women with ER-positive breast cancer[8].

In terms of a predictive value for Ki67, Dowsett *et al*[21] measured the expression of Ki67 in tumor biopsy samples taken before and after 2 wk of presurgical endocrine treatment in postmenopausal hormone receptor-positive breast cancer. They showed that a change in Ki67 labeling index between levels before and after 2 wk of endocrine treatment was significantly associated with clinical response. On the other hand, we demonstrated that Ki67 level in a tumor biopsy before treatment with the neoadjuvant aromatase inhibitor exemestane did not correlate with response to the therapy[9,22].

In contrast, in premenopausal women, overall tumor response was better in patients who had a baseline Ki67 index of ≥ 20% compared with those whose baseline Ki67 index was < 20% in a study of patients treated with neoadjuvant anastrozole or tamoxifen who also received goserelin for 24 wk[14]. It is possible that Ki67 may be positively stained in ER-positive breast cancer cells with estrogen-dependent growth, and that neoadjuvant endocrine treatment may be effective for Ki67-positive, estrogen-dependent tumor cells in premenopausal women.

CONCLUSION

Clinical roles of PgR and Ki67 in ER-positive breast cancer differ between pre- and postmenopausal women. Of the available multigene assays, PgR and Ki67 are included in Oncotype DX and PAM50, and genes related to ER-signaling are included in EndoPredict. Care should be taken when these assays are introduced for premenopausal women, because most studies involved in the development of multigene assays for ER-positive breast cancer were performed in postmenopausal women. We previously analyzed genetic and environmental factors, endogenous hormones and growth factors to identify risk factors for ER-positive breast cancer, and showed that risk factors differ between women of different menopausal status[23]. We therefore suggest that the mechanisms of development and estrogen-dependent growth of ER-positive breast cancer might differ according to menopausal status.

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REVIEW

Adjuvant chemotherapy for rectal cancer: Is it needed?

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Abstract

Adjuvant chemotherapy has become a standard treatment of advanced rectal cancer in the West. The benefits of adjuvant chemotherapy after surgery alone have been well established. However, controversy surrounds the use adjuvant chemotherapy in patients who received preoperative chemoradiotherapy, despite it being recommended by a number of international guidelines. Results of recent multicentre randomised control trials showed no benefit of adjuvant chemotherapy in terms of survival and rates of distant metastases. However, concerns exist regarding the quality of the studies including inadequate staging modalities, out-dated chemotherapeutic regimens and surgical approaches and small sample sizes. It has become evident that not all the patients respond to adjuvant chemotherapy and more personalised approach should be employed when considering the benefits of adjuvant chemotherapy. The present review discusses the strengths and weaknesses of the current evidence-base and suggests improvements for future studies.

Key words: Rectal cancer; Adjuvant chemotherapy

Core tip: Adjuvant chemotherapy for rectal cancer is a contentious issue despite its widespread use. Recent randomised controlled trials have shown no benefit in survival of adjuvant chemotherapy in patients treated with preoperative chemoradiotherapy. It is becoming evident that not all patients benefit from adjuvant chemotherapy and identification of these patients should be the focus of future studies. The present review discusses the current evidence-base for adjuvant chemotherapy in rectal cancer and provides directions for future research.

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INTRODUCTION

The role of adjuvant chemotherapy in advanced rectal cancer in combination with preoperative chemoradiotherapy is controversial. Colorectal cancer is a major cause of morbidity and mortality worldwide. It is the third most common cancer worldwide and the fourth most common cause of cancer-related death[1]. Rectal cancer is defined as carcinoma arising in the distal 15 cm from the anal verge. It is estimated that approximately 40000 new cases of rectal cancer were diagnosed in the United States and 14226 in the United Kingdom in 2014[2,3]. Surgical treatment is the cornerstone of curative therapy for rectal cancer. Indeed, patients with early disease (stage Ⅰ, T1/2, node negative) can be effectively treated with surgical resection and 90% are expected to survive at 5 years[4]. Therapeutic approach and prognosis differs significantly in more advanced rectal cancers (stage Ⅱ and Ⅲ, T3/4, node negative or positive). Local recurrence rates are significantly higher with more advanced lesions compared to early disease (13% *vs* 5%) and 5-year survival is markedly decreased (35% *vs* 90%)[4,5]. As a result, a more aggressive approach combining radical surgical resection with total mesorectal excision (TME), radiotherapy and chemotherapy is used to treat locally advanced rectal cancers. Neoadjuvant chemoradiotherapy has now become a standard practice in the United States and Europe after the seminal German rectal cancer trial, which showed lower local recurrence rates in neoadjuvant chemoradiotherapy group compared to postoperative chemoradiotherapy[6]. Neoadjuvant chemoradiotherapy has led to an increase in sphincter sparing operations and better quality of life as a result of pre-operative downstaging, and decreased risk of local recurrence[7].

Although mortality and local recurrence rates have improved dramatically over the past decades as a result of more accurate preoperative staging modalities [magnetic resonance imaging (MRI), endoscopic ultrasound] and surgical techniques (TME), the rate of systemic relapse is still unacceptably high and contributes significantly[8]. About a third of patients with advanced rectal cancer will eventually develop distant metastases[6]. In order to prevent this, postoperative adjuvant chemotherapy has been employed in the management of locally invasive treatment of rectal cancer and is now incorporated into most treatment protocols in the west. Various national and international guidelines (National Comprehensive Cancer Network, American Society of Clinical Oncology, European Society of Medical Oncology, National Institute of Clinical Excellence) recommend postoperative chemotherapy with either capecitabine or 5-FU for a total of 6 mo for stage Ⅱ and Ⅲ rectal cancers irrespective of surgical pathology results[9]. Despite the widespread use of this approach, the evidence for beneficial effects of postoperative chemotherapy is conflicting. Indeed, the long-term results (10 years of follow-up) of the European Organisation for Research and Treatment of Cancer (EORTC) 22921 randomised trial published in 2014 showed no benefit of postoperative adjuvant chemotherapy after preoperative chemoradiotherapy prompting the authors to question the validity of current recommendations[10]. Whether or not postoperative chemotherapy should be given is an important clinical dilemma for healthcare professionals, as chemotherapy is associated with significant systemic toxicity, which may lead to diminished quality of life[11]. The present review provides an update on the current evidence-base for treatment of rectal cancer with adjuvant chemotherapy, discusses the strengths and pitfalls of recent research and suggests improvements for future studies.

DIFFERENCES BETWEEN COLON AND RECTAL CANCER

Current recommendations for adjuvant chemotherapy treatment of rectal cancer are based on the evidence, which is largely extrapolated from studies in colon cancer[12-14]. However, it is now known that clinical course and biology of colon and rectal cancers differ significantly. Rectal cancers have distinct gene expression profile, fewer BRAF mutations and less microsatellite instability[15-17]. Furthermore, colon and rectum posses distinct embryological origins as well as anatomical and physiological characteristics. Clinically, rectal cancers have a worse prognosis in the early stages of disease, but longer survival in more advanced stages compared to colonic tumours of the same stage[18]. Finally, it is more difficult to achieve complete resection of rectal cancers with circumferential margin involvement, due to multi organ involvement, compared to colonic cancers[19]. As a result, it is scientifically justifiable to consider colonic and rectal cancers as distinct diseases and therefore the benefits of adjuvant chemotherapy cannot be assumed to be equal in both conditions.

POSTOPERATIVE CHEMOTHERAPY IN COMBINATION WITH SURGERY ALONE

The value of postoperative chemotherapy in patients treated only with curative surgery has been investigated in a large number of trials. The Cochrane systematic review and meta-analysis (2012) of 21 RCTs comparing postoperative chemotherapy with observation alone found significant improvement in both overall (HR = 0.83, 95%CI: 0.76-0.91) and disease-free survival (HR = 0.75, 95%CI: 0.68-0.83)[20]. Data, pooled from almost 10000 patients, showed that 5-FU based postoperative chemotherapy was associated with risk reduction of 17% and 25% in overall and disease-free survival respectively. Only 5 out of 21 trials showed significantly

positive results, which implies that large numbers of study participants are needed to discern a small, but clinically important, difference. Of note, nine trials were conducted in Japan. Despite considerable differences in populations and treatment practices of rectal cancer in the west and in Asian countries (infrequent use of neoadjuvant chemoradiotherapy and different surgical technique in Japan), the authors of the Cochrane review found similar results both for Western and Japanese studies. It is unclear which groups of patients benefit most from chemotherapy, as only three trials reported results based on TNM stage. The QUASAR trial (*n* = 3239, 948 with rectal cancer) found significantly prolonged overall and disease survival in patients with stage Ⅱ (node negative) disease[12]. In contrast, a subgroup meta-analysis of three trials, which included patients with stage Ⅲ disease showed no significant improvement in overall survival, but longer disease-free survival[21-23].

The results should be interpreted with caution as the heterogeneity of the studies was high, most likely due to variable TNM stages (Duke's stages A to C). In addition, the studies were conducted over the course of the past three decades, during which surgical and oncological treatment practices have changed considerably. Indeed, there is an argument that postoperative chemotherapy without preoperative treatment was only found beneficial in older studies not employing TME surgery[24]. No RCTs in the TME era have evaluated the value of postoperative chemotherapy and are unlikely to be performed as neoadjuvant treatment has become a "gold standard" approach. Finally, postoperative radiotherapy was administered alongside chemotherapy in some of the studies, hence individual contribution of chemotherapy to increased survival is difficult to determine.

POSTOPERATIVE CHEMORADIATION AFTER SURGERY ALONE

Therapeutic utilisation of the synergistic effects of radiation and chemotherapy has dominated the treatment of cancer for many decades. The benefits of chemoradiation for rectal cancer was established in a number of trials (NSABP R-01, GITSG-7175, NCCTG-794751, GITSG-7180) in 1980s and early 1990s and is now a recommended optimal treatment modality in patients undergoing curative surgical resection[25-28]. The GITSG-7175 study (*n* = 227) was the first trial to show lower recurrence rates (33% *vs* 55%), but no effect on overall survival in patients treated with radiotherapy and fluorouracil with semustine (methyl-CCNU) compared to surgery alone and had to be terminated prematurely as a result of these findings[25]. The following NCCTG trial randomised 204 patients to either chemoradiation (FU-semustine) or radiotherapy. In contrast to GITSG trial, chemoradiotherapy was found to be associated with significant reduction (46%) in cancer related deaths compared to radiotherapy alone[27]. Based on these studies National Institute of Health in 1990 produced the guidance recommending that all rectal cancers with stages Ⅱ and Ⅲ should be treated with a combined pelvic irradiation and concomitant chemotherapy[29].

To date, only the GITSG-7175 trial compared postoperative chemotherapy alone *vs* chemoradiotherapy and found no significant difference in survival and local recurrence rates[25]. The results of the NASPB R01 trial (*n* = 555) showed that chemotherapy, when compared to surgery alone or radiotherapy, is associated with significantly prolonged disease-free survival[28]. Since postoperative radiotherapy has not been shown to prolong survival in rectal cancer, it is reasonable to believe that chemotherapy when combined with radiotherapy is responsible for reducing the risk of systemic dissemination of rectal cancer. The seminal trial, which compared preoperative with postoperative chemoradiation showed that patients achieve significantly better local control and have lower levels of systemic toxicity, although overall survival is similar in both approaches[6]. As a result of the findings of this trial, preoperative chemotherapy has gradually become a mainstay approach to treatment of locally advanced rectal cancer.

POSTOPERATIVE CHEMOTHERAPY AFTER NEOADJUVANT (CHEMO)RADIOTHERAPY AND SURGERY

Although postoperative chemotherapy with or without radiotherapy prolongs survival in patients treated with surgery alone, the evidence is much more conflicting in the setting of neoadjuvant treatment. Since majority of the patients in the West nowadays receive neoadjuvant chemoradiotherapy, the most pertinent question regarding the benefit of postoperative chemotherapy remains unanswered by the studies described above. In light of several systematic reviews reporting no benefit of neoadjuvant chemoradiotherapy when compared to radiotherapy alone in terms of disease free and overall survival, the role of postoperative chemotherapy has come into question[30,31]. Five recent European trials (CHRONICLE, QUASAR, EORTC 22921, PROCTO-SCRIPT, I-CNR-RT) enrolling 3143 patients with stage Ⅱ and Ⅲ rectal cancer investigated the benefits of postoperative chemotherapy after neoadjuvant chemoradiotherapy and surgery (Table 1)[10,12,32-34]. Four out of five trials reported negative results and only QUASAR study found significantly increased survival in the postoperative chemotherapy group. EORTC 22921 trial (*n* = 1011) employed 2 × 2 factorial design comparing the effectiveness of postoperative 5-FU and leucovorin based chemotherapy after preoperative chemoradiation or radiotherapy alone[10]. No difference in overall and disease-free survival was reported at 5 and 10 years of follow up. In the Italian trial (I-CNR-RT), 635 patients were treated with preoperative chemoradiotherapy

Table 1 Trials comparing adjuvant chemotherapy with observation after neoadjuvant treatment

	Sample size	Accrual period	Total mesorectal excision	Preoperative treatment	Adjuvant treatment	Adherence (%)	Overall survival (adjuvant *vs* observation)	Disease-free survival (adjuvant *vs* observation)	Local recurrence (adjuvant *vs* observation)
EORTC 22921	1011	1993-2003	36.80%	25 doses of 1.8 Gy and fluorouracil-based chemotherapy	Four courses every 3 wk of fluorouracil and folinic acid	42%	51.8% *vs* 48.4%, $P = 0.32$	47% *vs* 43.7%, $P = 0.29$	11.7% *vs* 11.8%
CHRONICLE	113	2004-2008	NR	45 Gy and fluorouracil-based chemotherapy	Six courses every 3 wk of oxaliplatin and oral capecitabine	48.10%	89% *vs* 88%, $P = 0.75$	78% *vs* 71%, $P = 0.56$	Not reported
PROCTOR-SCRIPT	437	2000-2013	All patients	25 doses of 1.8-2.0 Gy and fluorouracil-based chemotherapy	Six courses of fluorouracil and folinic acid OR 12 courses of fluorouracil and folinic acid OR eight courses every 3 wk of oral capecitabine	73.60%	80.4% *vs* 79.2%, $P = 0.73$	62.7% *vs* 55.4%, $P = 0.13$	7.8% *vs* 7.8%, $P = 0.69$
I-CNR-RT	634	1992-2001	NR	25 doses of 1.8 Gy and fluorouracil-based chemotherapy	Six courses of fluorouracil and folinic acid	58.50%	70% *vs* 69.1%, $P = 0.77$	62.8% *vs* 65.3%, $P = 0.88$	4.5% *vs* 6.4%
QUASAR	3239 (948 with rectal cancer)	1994-2003	NR	Radiotherapy (21%)	Thirty doses of intravenous FU with high or low dose folinic acid	58.00%	HR = 0.8 (0.6-1.07)[1]	HR = 0.69 (0.51-0.94)[1]	19.8% *vs* 27.2%

[1]Hazard ratios were obtained from Cochrane review by Petersen *et al*[20]. HR: Hazard ratios; FU: Fluorouracil; NR: Not reported.

and then were randomised into observation and postoperative chemotherapy groups[33]. The investigators found no difference in 5-year survival and the distant metastases rates. PROCTO-SCRIPT trial ($n = 437$) patients treated with preoperative chemoradiotherapy were randomised into observation and treatment arms, which consisted of 5-FU plus leucovorin or capecitabine regimes[34]. The trial was stopped prematurely due to slow accrual and showed no benefit of postoperative chemotherapy in terms of overall survival. Another trial (CHRONICLE, $n = 112$), which was also terminated early due to slow accrual, found no survival advantage in patients treated postoperatively with capecitabine and oxaloplatin (XELOX)[32]. QUASAR trial was the only study to show borderline significant benefit of adjuvant chemotherapy after preoperative radiotherapy, however only 21% of patients with rectal cancer or both (rectal/colon) had radiotherapy[12].

In all of the studies above, adjuvant chemotherapy was associated with only marginal benefit, which was not statistically significant. None of the trials were large enough to detect a 5% difference in 5-year survival, hence the likelihood of type Ⅱ error was high[35]. As a result, Breugom *et al*[36] performed a meta-analysis of available studies using patient-level data. Unfortunately, the authors were not able to obtain the data from the QUASAR trial investigators. The analysis of 1196 patients with stage Ⅱ and Ⅲ rectal cancer with R0 resection showed no significant effect of adjuvant chemotherapy on overall survival, disease-free survival and distant metastases. In subgroup analysis, patients with tumours located 10-15 cm from the anal verge seemed to benefit from adjuvant chemotherapy as disease-free survival was significantly prolonged (HR = 0.59, 95%CI: 0.40-0.85; $P = 0.005$) and rates of distant metastases were lower (HR = 0.61, 0.40-0.94; $P = 0.025$). There was no survival difference between stages Ⅱ and Ⅲ.

A meta-analysis performed by Petrelli *et al*[37], which included 16 randomised and non-randomised studies (a total of 5457 patients) found that overall adjuvant chemotherapy had significantly positive effects on disease-free and overall survival and distant metastasis rates. However, the validity of the results is limited due to significant bias of non-randomised studies. Indeed, in stratified analyses significant benefit was observed only in the non-randomised studies. Study participants who received chemotherapy were often younger, had node negative disease and showed good response to preoperative chemotherapy. In addition, median follow up rates were often shorter than 5 years, which could have exaggerated overall and disease-free survival in the short term.

The findings of these studies beg two questions question: Are current recommendations for adjuvant chemotherapy in rectal cancer valid? Or, are the findings of the

studies reliable enough to change current practice?

POTENTIAL PITFALLS OF THE CURRENT EVIDENCE

Although the RCTs described above are generally held to have robust designs, there are some important considerations to be made when interpreting the results. Poor adherence to postoperative chemotherapy is a well-recognised problem in the treatment of colorectal cancer. Of the patients assigned to the adjuvant chemotherapy group in the EORTC 22921 trial 25% did not start the adjuvant treatment, with similar figures in other studies. The numbers are even smaller for completion rates of chemotherapy with only around half of the patients fully complying with the treatment. Although this may reflect a real life scenario, it is pertinent to determine the effects of optimal chemotherapy treatment so that clinicians and patients can make the informed decision regarding the need for adjuvant chemotherapy. Breugom *et al*[34,36] argued that the results of the trials could have not been affected by poor adherence as PROCTOR-SCRIPT trial showed no benefit of chemotherapy for patients who completed all cycles. Unfortunately the number of patients in this group (n = 159) is too small to detect the clinically meaningful difference.

Another important consideration is a change of surgical practices over long accrual periods. Most trials commenced recruitment in the early 1990s (EORTC 22921, I-CTR-RT, QUASAR). Surgical practices have changed considerably since then and the type of surgery patients received in the trials poorly reflect current standards. For instance, in the EORTC 22921 trial TME was performed in only 36.8% of patients, which contrasts with the contemporary practices where TME is performed virtually in all patients with locally advanced rectal cancers[38]. Furthermore, abdominoperineal resection rate was 47.2% in the intervention arm in the CHRONICLE study, which is significantly higher proportion compared to the United Kingdom National Bowel Cancer Audit Programme (24%)[39]. These deviations from current treatment practices raise concerns about the applicability of the study findings to today's management of rectal cancer.

One of the most important shortcomings of the present studies is the use of inadequate imaging modalities. All of the RCTs relied on CT staging before the commencement of neoadjuvant treatment. Endoscopic ultrasound was only performed in 67% of patients in the EORTC 22912 trial and only in a selected proportion in I-CNR-RT study. The accuracy of the CT based-TNM staging is not perfect and the risk of overstaging is high[40]. Hence, it is likely that many patients were over-treated. Furthermore, CT does not enable accurate assessment of circumferential resection margin (CRM), which is an independent prognostic factor for disease-free survival[41-43] (Figure 1). The best modality to assess the extent of CRM is MRI, however no chemotherapy trials have reported the use of MRI[44] (Figure 1).

Furthermore, lymph node status was determined using pathological staging. Earlier studies have indicated that preoperative chemoradiotherapy may reduce the number of lymph nodes available for pathological examination and thus may affect the accuracy of staging[45-49]. There is a theoretical risk that some patients with metastatic lymph nodes are not identified on pathological staging and are at risk of systemic dissemination[49]. In particular, proximal node involvement carries a significant risk of distant metastasis[49]. Advanced imaging modalities, such as PET and MRI may enable accurate assessment of lymph node involvement before neoadjuvant chemoradiotherapy and would subsequently guide clinicians in deciding whether or not adjuvant therapy is necessary (Figure 2).

The timing of adjuvant chemotherapy may also have a considerable effect on survival outcomes and has been largely overlooked in the present studies. Several meta-analyses showed that the longer the chemotherapy is delayed the shorter survival is in patients with colorectal cancer[50,51]. One of the reasons why colon cancer responds to adjuvant chemotherapy and rectal does not may be prompt administration of adjuvant chemotherapy[9]. Stoma and prolonged preoperative radiotherapy especially in combination with chemotherapy for rectal cancer means that adjuvant chemotherapy may not start until months later. Adverse consequences of delayed chemotherapy are also supported by animal studies, in which surgery was shown to increase the number of circulating neoplastic cells and promote metastatic growth[52]. In addition, surgery has been shown to enhance the production of oncogenic growth factors, such as transforming growth fact - alpha[53,54].

Finally, the most informative analysis of these trials by Breugom *et al*[34,36] is not without limitations. Out of 2195 patients available from four trials, only 1196 were included. These included patients only with stage Ⅱ and Ⅲ disease who had R0 resection, hence the meta-analyses does not address the question whether responders to neoadjuvant chemoradiotherapy achieve any benefit from adjuvant chemotherapy (see below). In addition, QUASAR, which was one of the largest trials and showed positive effects, was not included in the analysis.

WHO MIGHT BENEFIT FROM POSTOPERATIVE CHEMOTHERAPY?

Several studies have suggested that not all the patients with rectal cancer benefit from adjuvant chemotherapy and that only certain groups may respond to treatment. The degree of bowel wall penetration and nodal involvement has been shown to be one of most important predictive factors for local relapse, distant metastasis and survival[4,5,55]. For example, in a pooled analysis

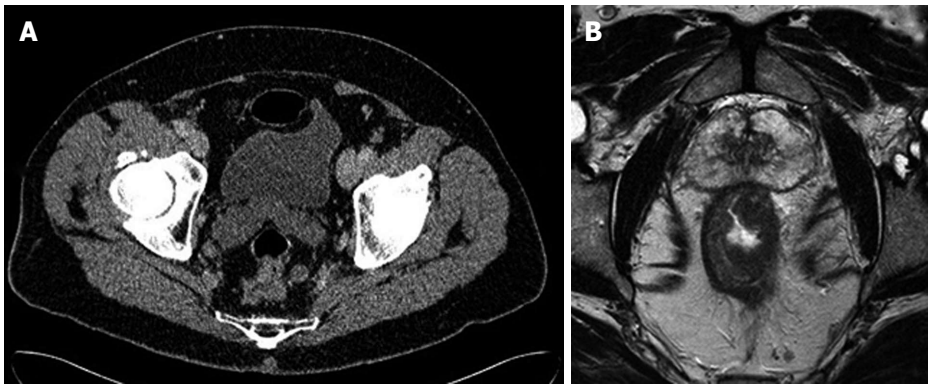

Figure 1 Computed tomography (A) and magnetic resonance imaging (B) of the T3 rectal cancer. Note poor quality of circumferential margin on the computed tomography scan compared to the magnetic resonance imaging.

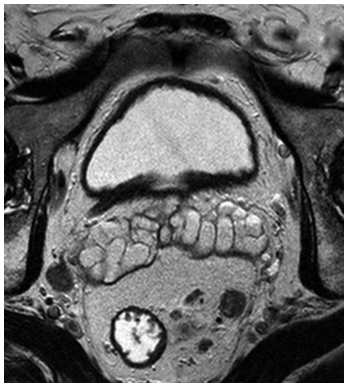

Figure 2 Magnetic resonance imaging of the T3 rectal cancer showing lymph node involvement.

of five randomised control trials in the United States, which included 3791 patients with rectal cancer, 5-year overall survival for T1-2N0 stage was 90%, for T3-4N0 60%, T4N1 30%[4]. Many studies have been conducted to investigate the benefits of chemotherapy in certain subgroups of patients, however the results haven been rather conflicting. Most of the evidence comes from post-hoc subgroup analyses of randomised control trials or retrospective/prospective non-randomised studies, hence is subject to inherent weaknesses of these designs.

The exploratory analysis of the early results (5 years of follow up) of the EORTC 22921 trial has showed that only patients downstaged to ypTN0-2 benefit from adjuvant chemotherapy, while patients with ypTN3-4 do not[56]. In line with this, two other studies by De Stefano *et al*[57] and Janjan *et al*[58] found that patients who responded to preoperative chemoradiotherapy benefited from adjuvant chemotherapy, however no benefit was observed in the non-responders group. Such observations also have sound scientific basis, since rectal cancers are highly heterogenous tumours and preoperative chemotherapy may enable to predict favourable tumour biology, which may respond to subsequent adjuvant chemotherapy.

On the other hand, there have been several reports to suggest that downstaged ypTNM0-2 tumours follow a more indolent course postoperatively and do not require additional chemotherapy. Three studies have shown that patients with good response to preoperative chemotherapy had excellent 5-year survival (90% survival) irrespective whether adjuvant chemotherapy was given or not[59-61]. Hence, additional chemotherapy may not be necessary and potentially harmful. This is also supported by the results of the long term outcomes of EORTC 22921 trial[10]. The investigators showed that although there appeared to be a survival advantage in patients with downstaged tumours in the short term, this benefit was transient and the survival curves equalised after 10 years.

Unfortunately, in a majority of patients a highly favourable response to preoperative chemotherapy is not observed and they are at greater risk of local and distant recurrence as well as shorter survival[62]. As a result, it appears logical to treat these patients aggressively with adjuvant chemotherapy[55,59,63-65]. Unfortunately, Breugom *et al*[36] in the meta-analysis of five trials (described above) showed no benefit of adjuvant chemotherapy neither in stage Ⅱ nor in stage Ⅲ, however there was no data available for stage 0 and Ⅰ disease. The differing results in the studies above may reflect variations in chemotherapy regimes used. Poor response to neoadjuvant treatment, which is usually fluoropyridine-based, indicates unfavourable tumour pathology and, unsurprisingly, administration of fluoropyridines during postoperative period may bring no benefit due to tumour resistance. In these cases, more aggressive combined therapy may have a role. A retrospective analysis of 160 rectal cancers with ypN0 stage showed that patients with T3-4 disease have significantly longer disease-free and overall survival if adjuvant FOLFOX (Oxaliplatin with fluorouracil and folinic acid) or XELOX (capecitabine with oxaliplatin) regimens were given, while those with T0-2 appeared to show no benefit from adjuvant chemotherapy[64]. Randomised controlled trials are needed to determine whether non-responders may benefit from a more aggressive adjuvant treatment.

Location of rectal cancer in relation to anal verge was also found to have significance when aiming to predict which patients may benefit from adjuvant chemotherapy. In the subgroup analysis, Breugom *et al* reported that tumours occurring 10-15 cm from the anal verge have longer disease-free survival if adjuvant chemotherapy is administered (HR = 0.59, 95%CI: 0.40-0.85, *P* = 0.005). No significant interaction between distance from the anal verge and treatment group was found for more distal tumours. The authors proposed that this observation might be as a result of the arbitrary definition of rectum and that tumours in proximal rectum are in fact biologically similar to colonic ones. Bujko *et al*[35] suggested several anatomical reasons why low lying rectal cancer may have poor prognosis compared to higher ones. The authors argued that higher proportion of low lying rectal cancers involve a circumferential margin. In addition, lower cancers receive both systemic and portal venous drainage and hence are at risk of systemic dissemination. Finally, internal iliac and obturator nodes are at risk of involvement in low lying rectal cancers, which are not routinely removed in the West.

ADJUVANT CHEMOTHERAPY AGENTS: PAST, PRESENT AND FUTURE

Fluoropyrimidine-based agents

Fluoropyrimidine-containing agents have formed the basis of adjuvant chemotherapy in rectal cancer. 5-FU can be administered either bolus or by continuous intravenous infusion. The NCCTG trial involving 660 patients with locally advanced rectal adenocarcinoma showed that protracted venous infusion of 5-FU (PVI FU) alongside pelvic irradiation was associated with significantly reduced distant metastases rate (31% *vs* 40%) and increased overall survival[66]. In contrast, a larger study (*n* = 1917) by Smalley *et al*[67] found no significant differences between three trial arms (5-FU bolus plus leucovorin, 5-FU bolus plus infusion, 5-FU only) (United States intergroup study). There appears to be limited evidence to favour PVI FU over simple bolus FU in rectal cancer bearing in mind higher costs, inconvenience and requirement for a central line.

An attractive alternative to PVI FU is an oral agent called capecitabine. Capecitabine requires 3-step enzymatic activation *in vivo*, one of which preferentially occurs in tumours, hence capecitabine offers a highly targeted approach. Trials, mainly investigating the effectiveness of capecitabine in the neoadjuvant setting, show non-inferiority to intravenous 5-FU regimens in terms of disease-free and overall survival and distant and local recurrences[68,69]. A phase Ⅲ German trial randomised 392 patients to receive either capecitabine or intravenous 5-FU in the perioperative period (231 patients received postoperative adjuvant chemotherapy). The results showed significantly lower distant metastases rates with capecitabine compared to 5-FU (19% *vs* 28%), however similar 5-year survival (76% *vs* 67%)[70]. Aside from higher risk of hand-foot syndrome, capecitabine offers a great substitute to intravenous 5-FU regimes and obviates the need for a central line and is already recommended by the National Comprehensive Cancer Network guidelines.

Oxaliplatin-based regimens

Oxaloplatin is a third-generation 1,2 diaminocyclohexane platinum analogue, which prevents replication and transcription of DNA. The MOSAIC and NSABP C07 trials showed significant improvement in overall survival in patients with advanced colon cancer[71,72]. Based on these encouraging results, several trials were carried out to determine the benefits of oxaliplatin in addition to standard fluopyridinamine-based regimens in rectal cancer (ACCORD12/0405-Prodige, CAO/ARO/AIO-04, ADORE, STAR-01, NSAPB R-04, PETACC-6)[73-78]. While some studies reported significant improvement in pathological response and disease-free survival[76,78], others found no superiority of oxaliplatin, but instead an increased risk of acute toxicity[74,75,77,79]. Only four trials reported the data on survival. Recently published results of ADORE trial showed significant improvements in 3-year disease-free survival in FOLFOX group (5-FU, oxaliplatin, leucovorin) compared to (5-FU and leucovorin) (71.6% *vs* 62.9%, HR = 0.657, 95%CI: 0.434-0.994; *P* = 0.047)[78]. Toxicity was more commonly seen in FOLFOX group, however there was no difference in frequency of grade 3 and grade 4 events. Similar results were reported in the CAO/ARO/AIO-04 trial, which showed significant increase in the proportion of patients achieving pathological complete response (17% *vs* 13%) and improved 3-year disease survival[76]. In contrast, interim results of PETACC-6 trial reported in a conference abstract did not show survival advantage in FOLFOX group[79]. CHRONICLE trial reported no benefit of oxaliplatin, however the study was considerably underpowered[32]. Three trials did not find improvement in pathological complete response (NSAPB R-04, ACCORD 12/0405-Prodige, STAR-01), however no data on survival were available. Although the evidence for oxaliplatin use in rectal cancer is limited, adjuvant chemotherapy incorporating oxaliplatin is widely used and is recommended by a number of international guidelines.

Irinotecan and biological agents

Irinotecan is a plant alkaloid, which inhibits DNA replication and repair by blocking topoisomerase Ⅰ. Although irinotecan has been used with success in metastatic colon cancer, no benefit was found for stage Ⅲ[80,81]. Only one trial investigated the benefits of irinotecan in rectal cancer[82]. The study recruited only 225 patients out of expected 3250 and was terminated because of the competing trial on bevacizumab. The investigators reported no benefit of addition of irinotecan to fluorouracil and leucovorin in neoadjuvant or adjuvant settings. Hence, currently irinotecan has no proven role

in treatment of rectal cancer.

Biological agents such as anti-VEGF agent, bevacizumab, and monoclonal antibodies, cetuximab and panitumumab, which target epidermal growth factor receptor have been successfully used in metastatic colon cancer in patients who failed on first line chemotherapy regimens[83-85]. Although approved by FDA, NICE currently does not support their use[86]. The role of bevacizumab in non-metastatic rectal cancer is unknown. The on-going phase Ⅱ BACCHUS trial is comparing FOLFOX with bevacizumab *vs* FOLFOXIRI with bevacizumab in the neoadjuvant setting in patients with locally advanced rectal cancer. However, the trial does not directly test the independent benefits of bevacizumab and its role in adjuvant setting is not under investigation.

FUTURE DIRECTIONS AND TRIALS IN PROGRESS

Unfortunately, a definite answer regarding the effectiveness of adjuvant chemotherapy is unlikely to be forthcoming in the near future. Most on-going trials compare different chemotherapeutic agent combinations or intensification regimes (PETACC-6, NSAPB R04, AERO-R98) and do not include an observation arm. Hence, the fundamental issue of whether or not adjuvant chemotherapy is effective is unaddressed. The only phase Ⅲ trial (NCT01941979) registered in the http://clinictrials.gov.uk website (accessed February 2015), which includes an observation arm is currently open and recruiting. The trial compares FOLFOX *vs* observation alone in patients with T3-4, N1, M0 who were treated with preoperative chemotherapy and showed poor response. The rationale of the study is based on the previous observations that only certain groups of patients with rectal cancer may benefit from adjuvant chemotherapy[57,87].

Since rectal cancer is a highly heterogenous disease, more trials are needed to take a targeted approach when evaluating the benefits of adjuvant chemotherapy. It is still unclear what role adjuvant chemotherapy has in patients who responded well to preoperative chemotherapy as the evidence is conflicting. Hence, ideally a separate trial investigating adjuvant chemotherapy is needed in this patient population. At the other end of the spectrum, the optimal management of patients who did not show improvement with preoperative chemoradiotherapy is also unclear. The use of adjuvant chemotherapy in non-responders appears to be unsupported by current evidence. However, there is scope for a more aggressive approach employing intensification regimens and combination treatments, including oxaliplatin and bevacizumab.

Reporting of the results based on stage may not be sensitive enough since there is high variability in prognosis within each TNM stage[88]. Valentini *et al*[89] produced nomograms based on the data from five major European RCTs on adjuvant chemotherapy in rectal cancer (n = 2795), which take into account a large number of clinical and pathological variables. Using these nomograms to stratify patients with rectal cancer into low, intermediate and high risk groups may help identify with high accuracy patient subgroups, which would benefit from adjuvant chemotherapy, however a randomised control trial is needed to determine their benefit.

Accurate clinical staging before and after administration of preoperative chemotherapy is vital to avoid over-staging and subsequent overtreatment. CT and EUS assessment is far from adequate and instead MRI should be employed. Particular areas of interest are circumferential margin involvement and lymph node status, as these are the most important predictors of poor survival[43,49].

THE ROLE OF BIOMARKERS

It has been increasingly recognised that all cancers in general, including rectal cancer, are highly heterogenous diseases requiring personalised therapies. Identification of reliable biomarkers could potentially aid clinical decision-making regarding the need for adjuvant chemotherapy. Many studies have identified dozens of biomarkers (microsatellite instability, p53, KRAS, BRAF, thymidylate synthase) in colon cancer and a 12-gene recurrence score assay (Oncotype DX Colon Cancer Assay) has been validated in the QUASAR trial as a reliable predictor for distant recurrence[90,91]. Whether similar assays can be used in rectal cancer is not known due to biological differences of colon and rectal cancer and requires separate validation. Biomarker analysis of the PROCTO-SCRIPT trial specimens is planned, which will hopefully help to identify patients who would benefit from adjuvant chemotherapy[34].

CONCLUSION

Adjuvant chemotherapy for rectal cancer has been a subject of controversy in recent years. The results of major trials, published in the last couple of years, do not support the use of postoperative chemotherapy after neoadjuvant chemoradiotherapy, however many clinicians throughout the world are understandably reluctant to abandon adjuvant chemotherapy. Concerns exist regarding the quality of the studies including inadequate staging modalities, out-dated chemotherapeutic regimens, non-TME surgical approaches and small sample sizes. It is becoming evident that not all patients with rectal cancer need adjuvant treatment. Identification of groups at risk using advanced imaging modalities, nomograms and biomarkers is the future of personalised treatment of rectal cancer. Hopefully, these questions will be answered in the near future. In the meantime, patients should be informed of benefits and risks of postoperative chemotherapy and the decision regarding the need for further treatment should be made

on individual basis.

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REVIEW

Therapeutic role of template-based lymphadenectomy in urothelial carcinoma of the upper urinary tract

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Abstract

Lymphadenectomy for urothelial carcinoma of the upper urinary tract has attracted the attention of physicians. The mapping study of lymphatic spread has shown that a relatively wide area should comprise the regional nodes for tumors of the right renal pelvis or the right upper two-thirds of the ureter. A prospective study showed that an anatomical template-based lymphadenectomy significantly improved patient survival in tumors of the renal pelvis. This benefit was more evident for patients with pT2 stage tumors or higher. The risk of regional node recurrence is significant reduced by template-based lymphadenectomy, which is likely to be associated with improved patient survival. The removal of lymph node micrometastases is assumed to be the reason for therapeutic benefit following lymphadenectomy. The number of resected lymph nodes can be used to assess the quality of lymphadenectomy, but not to determine the extent of lymphadenectomy. The guidelines currently recommend lymphadenectomy for patients with muscle-invasive disease, even though the current recommendation grades are still low. The present limitation of lymphadenectomy is the lack of standardization of the extent of lymphadenectomy and the randomized trials. Further studies are warranted to collect the evidence to support lymphadenectomy.

Key words: Lymphadenectomy; Lymph node excision; Urothelial carcinoma; Treatment outcome; Therapeutic uses; Diagnosis; Guideline

Core tip: The role of lymphadenectomy in urothelial carcinoma of the upper urinary tract had examined. A prospective study showed that anatomical template-based lymphadenectomy significantly improves patient survival in tumors of the renal pelvis. This benefit is demonstrated more clearly for patients with pT2 tumors or higher. The risk of regional node recurrence is significant reduced by template-based lymphadenectomy, which is likely to be associated with improved patient survival. The guidelines currently recommend lymphadenectomy for patients with muscle-invasive disease. Further studies are warranted to

collect the evidence to support lymphadenectomy.

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INTRODUCTION

About 20%-30% of patients with urothelial carcinoma develop lymphatic metastases, and thus, it is known to confer a high risk of developing lymphatic metastases[1,2]. Thus, controlling lymphatic spread may be an important strategy to improve patient survival. Lymphadenectomy may be a possible strategy for surgically treating cancer that spread to the lymph nodes. The standard surgical treatment for muscle-invasive bladder cancer is radical cystectomy[3]. Concomitant lymphadenectomy provides a better outcome than no lymphadenectomy, and an extension in the lymphadenectomy template may possibly result in higher patient survival[4,5]. Thus, guidelines currently recommend lymphadenectomy as an integral part of radical surgery for bladder cancer[3].

Most carcinomas arising from the upper urinary tract are pathologically urothelial carcinomas, which are similar to bladder cancer. It is well known that there is a high risk of metastases to the lymph nodes in urothelial carcinomas of the upper urinary tract (UCUT)[6,7]. Moreover, stage and grade migration toward more aggressive disease has been reported in UCUT[8]. Thus, one can speculate that controlling metastases to the lymph nodes is more important in UCUT.

In this review article, we summarize the current understanding of the role of lymphadenectomy in UCUT. Unfortunately, the evidence regarding lymphadenectomy in UCUT is small as compared with that for bladder cancer. A recent study by the cancer registry shows that lymphadenectomy is rarely performed[9]. In addition, patient survival did not improve with radical nephroureterectomy over a period of 18 years[10]. The role of lymphadenectomy needs to be discussed to improve the outcome of surgery.

THE HISTORY OF LYMPHADENECTOMY IN UCUT

The high incidence of lymphatic metastases in UCUT was reported as early as the 1970s[6,7]. Thus, the inclusion of lymphadenectomy as a standard procedure was suggested for radical nephroureterectomy indications[11]. However, the role of lymphadenectomy was not examined sufficiently until the 1990s because UCUT is a very minor disease among malignancies[12].

In the 1990s, 2 studies shed new light on the importance of lymphadenectomy. Komatsu *et al*[13] reported the outcomes of relatively wide lymphadenectomy. Lymph node metastases that were pathologically confirmed by lymphadenectomy (pN0), were not significantly associated with higher patient survival than those with pathologically confirmed lymphatic metastases (pN+). This result supports the use of lymphadenectomy for staging. Another study by Miyake *et al*[14] showed that lymphadenectomy improved survival in selected patients without lymph vessel invasion. However, the small number of patients in these studies precluded widespread discussion. Thereafter, no new information regarding the benefits of lymphadenectomy was available until 2007.

THE EXTENT OF LYMPHADENECTOMY

In the 1980s, some investigators examined the primary sites of lymphatic metastases in UCUT[7,15,16]. Their results showed that metastases spread primarily to the renal hilar, abdominal para-aortic, and paracaval nodes from the renal pelvis and to the abdominal ureter and the intrapelvic nodes from the distal ureter. Current descriptions in the Union for International Cancer Control TNM classification is are based on results reported more than 30 years ago[17]. However, the location or laterality of primary tumors was not taken into account when considering the anatomical extent of the regional nodes. Therefore, the aforementioned results could not be used to determine the extent of lymphadenectomy in clinical practice.

In 2007, we conducted more detailed mapping studies of lymph nodes. In this study, we examined 42 patients with lymph node metastases confirmed by pathological examination of surgical specimens or radiological methods[18]. Sites of primary nodal metastases were identified according to the location of the tumors, for example, the renal pelvis, the upper and middle ureter, and the lower ureter. Our results showed that primary metastatic sites were located in a larger area than previously thought for tumors of the right renal pelvis and the upper two-thirds of the right ureter. We reanalyzed the pattern of lymphatic metastases by increasing the number of the patients with lymph node metastases to 75, but the results were similar (Table 1)[19]. In tumors of the right renal pelvis, lymphogenous metastases spread primarily to the right renal hilar, paracaval, retrocaval, and interaortocaval nodes. Primary metastatic sites in right upper and middle ureter tumors also include the right renal hilar, retrocaval, and interaortocaval nodes. Tumors of the left renal pelvis or the left upper/middle ureter primarily metastasized to left renal hilar and para-aortic nodes. The lower boundary of the metastatic sites was at the level of the inferior mesenteric artery for tumors of the renal pelvis and at the aortic bifurcation for tumors of the upper and middle ureter. Primary metastatic sites for tumors of the lower ureter included the ipsilateral common iliac, external iliac, obturator, and internal iliac

Table 1 The incidence of primary nodal involvement in each lymph node sites according to the location of the tumor in urothelial carcinomas of the upper urinary tract

Location of the primary tumor (No. of patients with nodal metastasis)		Ipsilateral						Ipsilateral				
		Suprahilar	Ipsilateral renal hilar	Para-caval	Retro-caval	Interaorto-caval	Para-aortic	Common iliac	External iliac	Obturator	Internal iliac	Presacral
Right	RP (22)	-	14 (84%)	8 (36%)	9 (41%)	3 (14%)	-	-	-	-	-	
	UU (3)	-	1 (33%)	-	1 (33%)	2 (66%)	-	-	-	-	-	
	MU (5)	-	-	-	1 (20%)	4 (80%)	-	-	-	-	-	
	LU (7)	-	-	-	-	-	-	4 (57%)	1 (14%)	5 (71%)	2 (29%)	1 (14%)
Left	RP (25)	-	20 (80%)	-	-	1 (4%)	11 (44%)	-	-	-	-	
	UU (0)	-	-	-	-	-	-	-	-	-	-	
	MU (5)	-	-	-	-	-	5 (100%)	-	-	-	-	
	LU (8)	-	-	-	-	-	-	4 (50%)	2 (25%)	3 (38%)	1 (13%)	

R-RP: Right renal pelvis; R-UU: Right upper ureter; R-MU: Right middle ureter; R-LU: Right lower ureter; L-RP: Left renal pelvis; L-UU: Left upper ureter; L-MU: Left middle ureter; L-LU: Left lower ureter.

nodes. Our first study did not reveal presacral nodes as a primary site, but a revised study showed that 14% of patients had primary metastases to this site.

Based on these results, we thought that nodal sites at more than 10% risk of metastasis, for example, regional lymph nodes, should be dissected. The proposed anatomical extent of lymphadenectomy is shown in Figure 1[19]. The suggested template for renal pelvic cancer is very similar to that for renal cell carcinoma, which is based on several studies[20]. For the right kidney, the paracaval, retrocaval, and precaval nodes should be included from the adrenal vein to the level of the inferior mesenteric artery, and for the left kidney, the para-aortic and pre-aortic nodes should be included from the crus of the diaphragm to the inferior mesenteric artery[20]. Interaortocaval nodes should always be removed despite the laterality of tumors when extended Lymphadenectomy (LND) is sought, but this is different from our results in which dissection of interaortocaval nodes can be ignored for tumors of the left renal pelvis. The template for lower ureteral cancer is also similar to that proposed for bladder cancer[21,22].

Our nonrandomized prospective study showed the therapeutic benefit of lymphadenectomy for tumors of the renal pelvis, confirming the rationale of this template[23]. However, our prospective study did not support the therapeutic role in ureteral cancer tumors. It remains to be determined whether the currently proposed anatomical template of ureteral cancer is appropriate.

Recently, another multi-institutional mapping study was reported, where a similar pattern of lymphatic metastases was observed for renal pelvic cancer. However, tumors below the crossing of the common iliac artery were more likely to spread cranially than expected, with an incidence of 33%-40%[24]. This might suggest that the template we propose for lower ureteral cancer is not adequate to cover primary metastatic sites. Further studies are warranted to standardize the extent of lymphadenectomy for UCUT.

DOES LYMPHADENECTOMY BENEFIT ACCURATE STAGING?

One of the major roles of lymphadenectomy is to provide accurate staging of lymphatic metastases. Lymphadenectomy could allow better stratification of patients to determine the indication of adjuvant therapy. In bladder cancer, lymphadenectomy has a role in staging. Extended lymphadenectomy reportedly improves staging accuracy because the incidence of pathological node metastases is increased by extending the extent of lymphadenectomy[5,25-27].

Several studies have examined the benefits of staging in UCUT. Komatsu *et al*[13] reported the role of relatively wide lymphadenectomy in 1997. Their results showed significantly higher cancer-specific survival (CSS) in patients without lymphatic metastases (pN0) as confirmed by lymphadenectomy compared to those with pathological node metastases (pN+), suggesting a role for wide lymphadenectomy in staging[13]. Roscigno *et al*[28] reported results for a similar extent of lymphadenectomy as Komatsu *et al*[13]. They compared patient survival between 3 groups, including patients without lymphatic metastases as confirmed by lymphadenectomy (pN0), those with pathological node metastases (pN+), and those without lymphadenectomy (pNx). Five-year CSS was highest in pN0 patients, moderate in pNx patients, and lowest in pN+ patients (73%

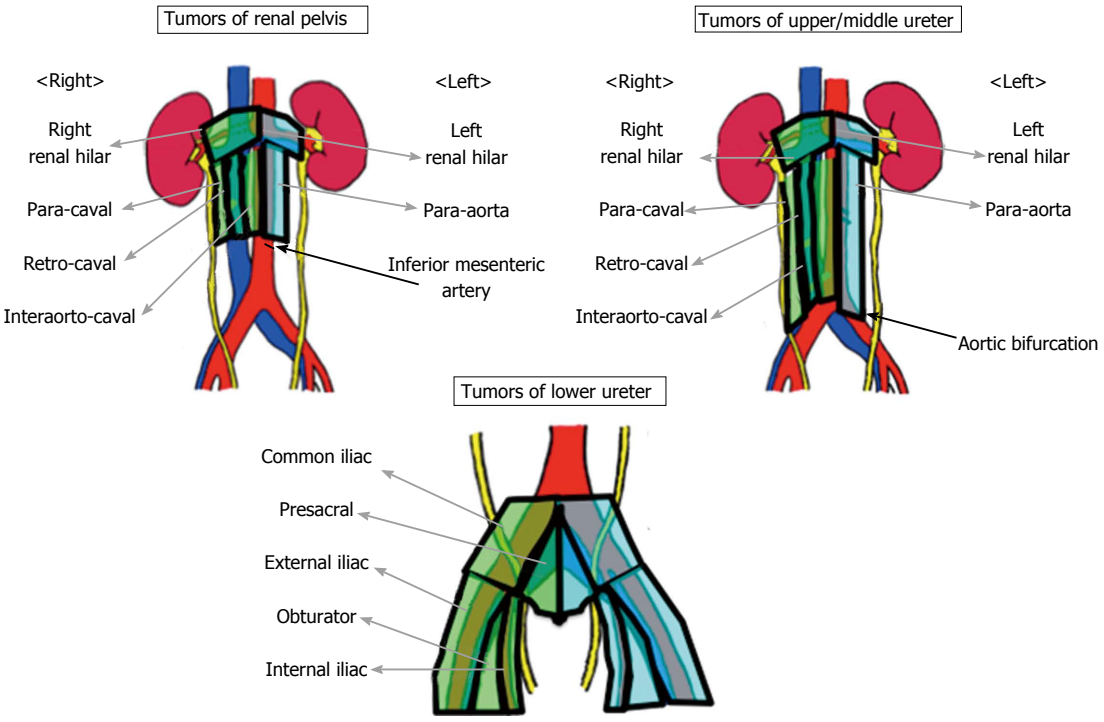

Figure 1 The extent of lymphadenectomy currently proposed for urothelial carcinoma of the upper urinary tract.

vs 48% *vs* 39%)[28]. Although there was no difference between pNx and pN0 patients (P = 0.476), the difference between pN0 and pNx patients was significant (P < 0.001). They concluded that lymphadenectomy is likely to provide better stratification of pN0 patients just like Komatsu *et al*[13].

Thereafter, several multi-institutional studies have been reported. The results are summarized in Table 2. Roscigno *et al*[29] conducted a multi-institutional study to further examine patient survival according to the lymph node status. CSS was stratified according to the nodal status in patients with a staging of pT1 or higher. Five-year CSS was 77% in pN0, 69% in pNx, and 35% in pN+. For patients with pT2 staging or higher, this difference was demonstrated more clearly (5-year CSS: 70% *vs* 58% *vs* 33%). Abe *et al*[30] also reported the results of a similar analysis. Recurrence-free survival was significantly higher in pN0 patients than in pNx patients with pT2 stage tumors or higher, but not in those with pT1. These 2 studies confirm the role of lymphadenectomy in stratifying patients with a favorable prognosis (pN0). This benefit is more prominent in those at the pT2 stage or higher. The extent of lymphadenectomy was described in these 2 studies, and they utilized a relatively wide template.

However, another 3 studies failed to demonstrate better stratification of pN0 by lymphadenectomy than that achieved without lymphadenectomy (pNx). On the other hand, these studies showed that lymphadenectomy could stratify patients with unfavorable prognosis by identifying pathological metastases to the lymph nodes (pN+)[31-34]. Lughezzani *et al*[31] collected the most number of patients by using for a population-based study by using a surveillance, epidemiology, and end results database. Lymphadenectomy could discriminate between pN+ patients with a poor prognosis, and pN0 or pNx patients. However, this benefit was limited to patients with a pT3 stage tumor or higher. Burger *et al*[32] also reported that stratification of pN+ patients with a significantly poor prognosis was observed only in locally advanced disease. Mason *et al*[33] also reported similar results to those by Lughezzani *et al*[31] and Burger *et al*[32]. Ouzzane *et al*[34] failed to demonstrate the benefit of staging in patients with a tumor of stage pT2 or higher, when examining 714 patients from multiple institutions in France. However, the extent of lymphadenectomy was not described in these 4 studies where the survival was similar between pN0 and pNx patients.

As mentioned above, there is a difference in the stratification of patients; pN0 stratification is better than pNx, and pN+ stratification is worse than pNx. One possible reason is the extent of lymphadenectomy. The latter 4 studies included all types of lymphadenectomy, whereas the first 2 studies had a relatively wide extent for dissection. We also examined the benefit of lymphadenectomy-based staging in our patient cohort. From 1988 to February 2015, we treated 314 nonmetastatic patients who underwent radical nephroureterectomy. Of these, 158 patients (53%) underwent lymphadenectomy, including 126 patients with lymphadenectomy based on the anatomical template (Figure 1, complete LND) and 42 where all regional sites were not dissected (incomplete LND). Our result was very similar to that reported by the others[29,30]. Five-year CSS, according to the status of lymph node metastases,

Table 2 Reports on staging benefit of lymphadenectomy in urothelial carcinoma of the upper urinary tract

Authors	Year	Institute	Template of LND	Subject	No. of patients	Results	Staging benefits	Ref.
Roscigno	2009	Multi	Not well described	≥ pT1	1130	5 yr-CSS: pN0 77% > pNx 69% (P = 0.032) > pN+ 35% (P < 0.001)	Yes	[29]
				≥ pT2	813	5 yr-CSS: pN0 70% > pNx 58% (P = 0.017) > pN+ 33% (P < 0.001)		
Abe	2010	Multi	Not well described	pT1	66	RFS: pN0 = pNx (P = 0.702)	Yes	[30]
				≥ pT2	227	RFS: pN0 > pNx (P < 0.001) = pN+ (P = 0.134)	in ≥ pT2	
Burger	2011	Multi	Not well described	Organ-confined	519	CSS: pN0 = pNx = pN+	Yes	[32]
							In locally advanced disease	
				Locally advanced	266	CSS: pN0 = pNx (P = 0.633) > pN+ (P < 0.001)		
Lughezzani	2010	Multi	Not described	pT1, pT2	1324	CSS: T1 pN0 = pNx (P = 0.4) = pN+ (P = 0.1)	Yes	[31]
						T2 pN0 = pNx (P = 0.8) = pN+ (P = 0.1)	In ≥ pT3	
				pT3, pT4	1382	CSS: T3 pN0 = pNx (P = 0.9) > pN+ (P < 0.001)		
						T4 pN0 = pNx (P = 0.3) > pNx (P < 0.001)		
Mason	2012	Multi	Not described	All patients	1029	OS: pN0 66.1% = pNx 66.0% (P = 0.617) > pN+ 22.3% (P < 0.01)	Yes	[33]
Ouzzane	2013	Multi	Not described	All patients	714	5 yr-CSS: pN0 81% = pNx 85% (P = 0.6) > pN+ 47% (P < 0.001)	Yes	[34]
							but in T1	
				≥ pT2	337	CSS: pN0 = pNx (P = 0.44) = pN+ (P < 0.15)		
TWMU	2015	Single	Well described	All patients	314	5 yr-CSS: pN0 84% > pNx 70% (P = 0.02) > pN+ 31% (P < 0.001)	Yes	-
				≥ pT2	212	5 yr-CSS: pN0 79% > pNx 59% (P < 0.007) > pN+ 31% (P < 0.004)		

LND: Lymphadenectomy; CSS: Cancer-specific survival; RFS: Recurrence-free survival; LNs: Lymph nodes; CompLND: Complete lymphadenectomy; DFS: Disease free survival; OS: Overall survival.

was 84.9% in pN0, 70.2% in pNx, and 31.5% in pN+ patients (Figure 2). The difference between the groups was statistically significant. This trend was demonstrated more clearly in patients with pT2 stage tumors or higher. Five-year CSS according to the status of lymph node metastases was 79.6% in pN0, 59.1% in pNx, and 31.5% in pN+ patients (Figure 2). Thus, we believe that the extent of lymphadenectomy influences the staging benefits.

Collectively, most studies agree that there are benefits from lymphadenectomy-based staging. In addition, this benefit is likely to be demonstrated more clearly in patients with advanced disease.

DOES LYMPHADENECTOMY IMPROVE SURVIVAL?

Retrospective study

In bladder cancer, extended lymphadenectomy where the cranial boundary of the template is at the level of aortic bifurcation has shown improvement in not only staging accuracy but also patient survival[4,5]. A therapeutic benefit of lymphadenectomy is expected in UCUT as well as bladder cancer because of histological similarity. However, no one had examined the role of lymphadenectomy in improving patient survival until 2007, except for Miyake *et al*[14] who showed that LND benefited only selected patients. The therapeutic benefits of lymphadenectomy are summarized in Table 3.

Three retrospective studies from single institutes were published in 2007. We identified an anatomical template of lymphadenectomy from the mapping study (Figure 1)[18]. Thus, we hypothesized that the extent of lymphadenectomy was an important factor that influences patient survival. In this study, we subclassified 169 patients into 3 groups, and compared the patient survival among groups[35]. The 3 groups include the patients for whom the regional nodes were all dissected [complete lymphadenectomy (CompLND)]; those in whom lymphadenectomy did not include all regional sites [incomplete lymphadenectomy (IncompLND)]; and those without lymphadenectomy (No-LND). CSS was lower in the No-LND group than in the CompLND or IncompLND groups, but the difference was not

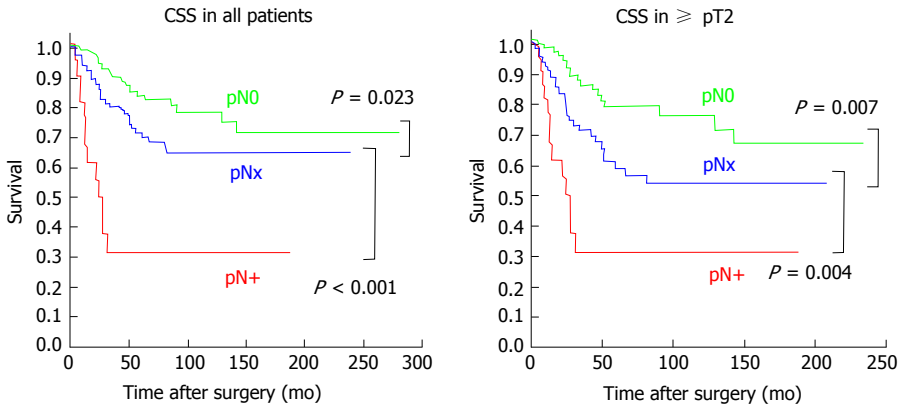

Figure 2 Benefit of staging lymphadenectomy by stratification of patients according to lymph node status in our institute. CSS: Cancer-specific survival.

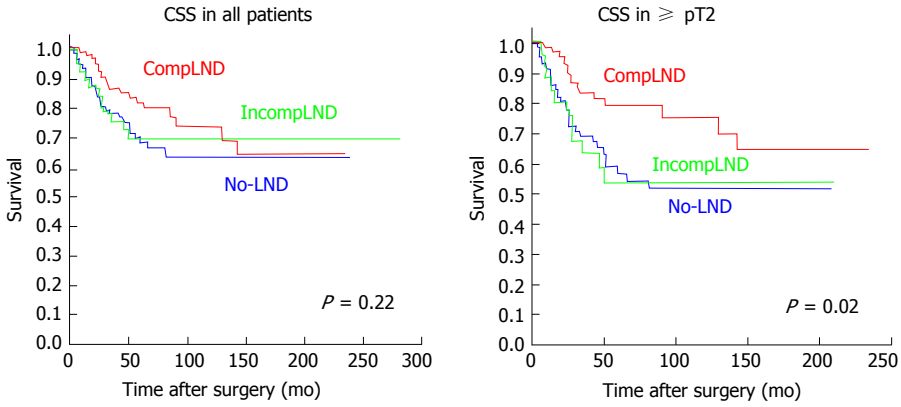

Figure 3 Therapeutic benefit of lymphadenectomy according to the extent of lymphadenectomy in our institute. LND: Lymphadenectomy; CSS: Cancer-specific survival.

statistically significant. However, for patients with pT3 stage tumors or higher, the survival rate increased incrementally from No-LND to IncompLND to CompLND. The difference between the CSS in the No-LND and CompLND groups, but not the IncompLND group, showed statistical significance. Multivariate analysis showed that CompLND was a significant independent factor for reducing the risk of cancer-specific mortality. Figure 3 shows the results from our current database, which includes 314 nonmetastatic patients, which is almost double that in our previous report. The results are similar to what we reported in 2007. A significant improvement in patient survival is observed in the CompLND group in patients with pT2 stage tumors or higher. In contrast, CSS in the IncompLND group was similar to that of No-LND even in patients with advanced stage cancer. Thus, our results suggest a therapeutic benefit of lymphadenectomy; however, lymphadenectomy should be performed based on the anatomical template.

Results from other retrospective studies have been reported. Brausi *et al*[36] reported the influence of relatively wide lymphadenectomy in 82 patients with pT2 stage tumors or higher. Lymphadenectomy included the following lesions: The para-aorta or vena cava between the renal hilum and the inferior mesenteric artery for tumors of the renal pelvis or the upper ureter; the para-aorta or vena cava between the renal hilum and the bifurcation of the common iliac artery for tumors of the mid-ureter; and the pelvic nodes on the ipsilateral side for lower ureteral tumors. The lymphadenectomy groups showed significantly higher disease-specific survival than those without lymphadenectomy in patients with pT2 stage tumors or higher (81.6% *vs* 44.8%, $P = 0.007$). Roscigno *et al*[28] also examined the influence of lymphadenectomy with an extent similar to that used by Brausi *et al*[36] on patient survival. Patients who underwent lymphadenectomy showed significantly higher CSS than those who did not undergo lymphadenectomy for advanced disease at the pT2 stage or higher (5-year CSS: 57% *vs* 40%, $P = 0.01$). These 2 studies from Italy also supported a therapeutic role for lymphadenectomy and emphasized the disadvantage of ignorance regarding lymphadenectomy.

Thereafter, multi-institutional retrospective studies were conducted to confirm the therapeutic benefit of lymphadenectomy. However, a major limitation of these multi-institutional studies is the lack of a standardized lymphadenectomy template among institutes and surgeons. Thus, we should carefully interpret these results. The largest study was reported by Roscigno *et al*[29], in which 1130 patients from 13 international

Table 3 Reports on therapeutic benefit of lymphadenectomy in urothelial carcinoma of the upper urinary tract

Authors	Year	Institute	Property	Template of LND	Subject	No. of patients	Survival results	Independent factors in Multivariate analysis?	Therapeutic benefit?	Ref.
Kondo	2007	Single	Retrospective	Clearly described	All patients	169	CSS: CompLND = IncompLND = No-LND (P = 0.06)	Yes: CompLND for CSS	Yes	[35]
					≥ pT3	88	CSS: CompLND > No-LND (P = 0.01)		In ≥ pT3	
Kondo	2012	Single	Retrospective	Clearly described	≥ pT2	191	5 yr-CSS: CompLND 77.9% > IncompLND 54.0% = No-LND 59.0% (P = 0.03)	Not determined	Yes	[23]
					≥ pT3	140	5 yr-CSS: CompLND 73.2% > IncompLND 43.7% = No-LND 47.3% (P = 0.01)		In ≥ pT2	
Brausi	2007	Single	Retrospective	Described	≥ pT2	82	DFS: RPLN 81.6% > No-LND 44.8% (P = 0.007)	Yes: RPLD for OS	Yes in ≥ pT2	[36]
Roscigno	2008	Single	Retrospective	Described	≥ pT2	132	5 yr-CSS: LND 57% > No-LND 40% (P = 0.01) pN0 72% > pNx 39% (P < 0.001)	Yes: LND and pN0 for CSS	Yes in ≥ pT2	[28]
					≥ pT2pN0	95	7 LNs > less than 7 (P < 0.001)	Yes: No. of LNs for CSS	Yes In ≥ 7 LNs removed	
Roscigno	2009	Multi	Retrospective	Not well described	≥ pT2	1130	5 yr-CSS: LND 66% = No-LND 69% (P = 0.23)	Yes: pN0 for CSS	No	[29]
Roscigno	2009	Multi	Retrospective	Not well described	≥ pT1pN0	412	5y-CSS: 8 LNs or more 84% > less than 8 73% (P = 0.038)	Yes: No. of LNs for CSS	Yes in ≥ 8 LNs removed	[37]
Abe	2010	Multi	Retrospective	Not well described	All patients	293	RFS: pN0 > pNx (P < 0.001) > pN+ (P = 0.004)	Yes: pNx of RFS	Yes	[30]
Burger	2011	Multi	Retrospective	Not well described	Organ-confined	519	CSS: pN0 = pNx	No	Yes but limited only in locally advanced disease	[32]
					Locally advanced	266	CSS: pN0 = pNx (P = 0.633)	Yes: pN0 for CSS in locally advanced		
Lughezzani	2010	Multi	Retrospective	Not described	All patients	2824	No; CSS is pN0 = pNx	No	No	[31]
Mason	2012	Multi	Retrospective	Not described	All patients	1029	OS: pN0 66.1% = pNx 66.0% (P = 0.617)	No	No	[33]
Ouzzane	2013	Multi	Retrospective	Not described	All patients	714	5y-CSS: pN0 81% = pNx 85% (P = 0.6) > pN+ 47% (P < 0.001)	No	No	[34]
					≥ pT2	337	CSS: pN0 = pNx (P = 0.44) = pN+ (P < 0.15)			
Kondo	2014	Multi	Prospective	Clearly described	Renal pelvis	90	≥ pT2 3 yr-OS: LND 86% > No-LND 48% (P = 0.01) 3 yr-CSS: LND 89% > No-LND 51% (P = 0.01) 3 yr-DFS: LND 77% > No-LND50% (P = 0.06)	Yes in CSS in > pT2	Yes in renal pelvic cancer in ≥ pT2	[23]
					Ureter	76	≥ pT2 3 yr-OS: LND 46% = No-LND 71% (P = 0.57) 3 yr-CSS: LND 54% = No-LND 71% (P = 0.99) 3 yr-DFS: LND 54% = No-LND 59% (P = 0.79)	No		

LND: Lymphadenectomy; LNs: Lymph nodes; CompLND: Complete lymphadenectomy; IncompLND: Incomplete lymphadenectomy; DFS: Disease-free survival; RPLD: Retroperitoneal lymph node dissection; RFS: Recurrence free survival; OS: Overall survival.

institutes were analyzed. Disappointingly, CSS was not significantly different between patients who underwent lymphadenectomy and those who did not (5-year CSS: 66% *vs* 69%, P = 0.23)[29]. Moreover, lymphadenectomy benefited pN0 patients. The number of lymph nodes removed significantly correlated with the improvement of CSS[37]. A

cutoff level of 8 lymph nodes improved CSS significantly (84% *vs* 73%, $P = 0.038$), and the number of lymph nodes removed was an independent factor for predicting CSS. Thus, it was suggested that lymphadenectomy should be performed to an adequate extent, which is in accordance with our principles for anatomical template-based lymphadenectomy[19,35].

Other studies were not a direct comparison between patients who did and did not undergo lymphadenectomy. They tried to find the therapeutic benefits of lymphadenectomy by comparing the survival of pN0 and pNx patients. This reflects the benefits of staging, but may also reflect therapeutic benefit. Abe *et al*[30] also reported improved CSS in pN0 patients compared to pNx patients with pT2 stage tumors or higher from multiple institutions. These studies also demonstrated that ignorance of lymphadenectomy (pNx) was an independent factor for predicting a poor patient outcome. Multivariate analysis by Burger *et al*[32] also showed an increased risk of recurrence and death in pNx patients with locally advanced disease. Thus, these 2 studies also support the therapeutic role of lymphadenectomy in patients with advanced disease.

However, 3 studies demonstrated no difference in patient survival between pN0 and pNx patients as mentioned in section 4[31,33,34]. In addition, multivariate analysis in a population-based study based on the surveillance, epidemiology, and end results database showed that omitting lymphadenectomy did not pose a disadvantage to patient survival[31]. They concluded that no therapeutic benefit was obtained from lymphadenectomy.

Thus, retrospective studies examining the therapeutic benefit of lymphadenectomy show large discrepancies among studies. One of the major reasons for this is the lack of standardization of the extent of lymphadenectomy. We need a prospective study to resolve this issue.

Prospective study

We conducted a prospective study in 2 Japanese institutes[23]. This study was initiated in 2006. At that time, we were not aware that the presacral lymph node was a regional node in lower ureteral cancer. Thus, dissection of presacral nodes was not necessary for inclusion in this study. In principle, template-based lymphadenectomy was performed at the time of radical nephroureterectomy in all patients irrespective of preoperative staging, except for patients over 75 years old or with significant comorbidities. Thus, this study was considered to be a nonrandomized prospective study. Lymphadenectomy was performed for 77 patients, while 89 patients did not undergo lymphadenectomy.

Figure 4 shows recurrence-free, cancer-specific, overall survival of patients. In patients with renal pelvic cancer, CSS and overall survival were significantly higher in the lymphadenectomy group compared to the no lymphadenectomy group, although the difference in recurrence-free survival was marginally significant. Multivariate analysis showed that template-based lymphadenectomy was a significant independent factor for reducing cancer mortality in patients with renal pelvic cancer. In contrast, lymphadenectomy did not improve patient survival in ureteral cancer. A similar trend was observed for patients with pT2 stage tumors or higher.

Thus, our bi-institutional, nonrandomized prospective study further supports a therapeutic benefit for lymphadenectomy in patients with renal pelvic cancer, but not in those with ureteral cancer. This study also confirms the rationale of using our anatomical lymphadenectomy template for renal pelvic cancer. Again, our prospective study failed to show the survival benefit of lymphadenectomy in ureteral cancer. However, our recent retrospective study shows that lymphadenectomy is also likely to improve survival in patients with upper/middle ureteral cancer, but not in those with lower ureteral cancer (prepared for submission). The template of lymphadenectomy for upper/middle ureteral cancer is similar to that for renal pelvic cancer. I believe that the benefit of lymphadenectomy will be confirmed in upper/middle ureteral cancer in the future. The reason why patients with lower ureteral cancer did not benefit from lymphadenectomy needs to be determined. Some possible explanations include an inadequate template and the higher malignant potential of lower ureteral cancer.

To the best of our knowledge, this is the only published prospective study that examines the role of lymphadenectomy. Another ongoing prospective study analyzed a preformed super-extended template[38]. They only reported the safety and feasibility of utilizing this template, not the patient survival. We definitely need a randomized trial to confirm the therapeutic benefit of lymphadenectomy.

Does lymphadenectomy reduce the risk of regional node recurrence?

There is a dearth of evidence to support the survival benefits of lymphadenectomy. One possible way of improving patient survival by lymphadenectomy may be the prevention of regional node recurrence.

Our prospective study shows significantly improved patient survival following anatomical template-based lymphadenectomy in renal pelvic cancer. In order to further examine the role of template-based lymphadenectomy, we analyzed how the extent of lymphadenectomy influences the recurrence pattern in renal pelvic cancer[39]. We collected the data of 180 patients with nonmetastatic (cN0M0) urothelial carcinoma of the renal pelvis from 2 institutions, and compared the sites of tumor recurrence between template-based lymphadenectomy, incomplete lymphadenectomy, and no lymphadenectomy. Recurrence in the regional nodes was significantly decreased in the complete template-based group (2.9%, 2/67) compared to the incomplete lymphadenectomy (18.1%, 4/22) and

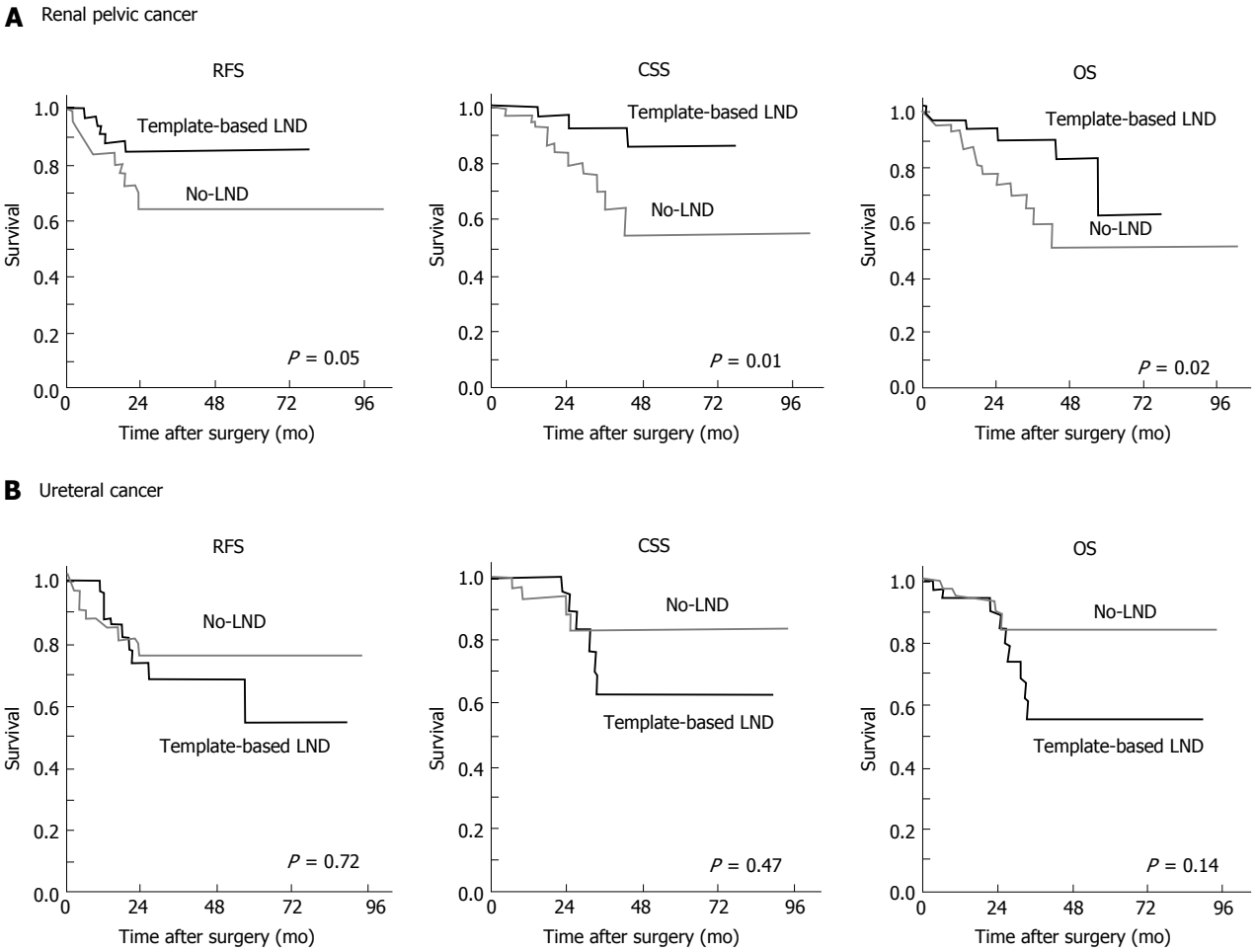

Figure 4 Patient survival in a non-randomized prospective study according to the location of the primary tumor. LND: Lymphadenectomy; CSS: Cancer-specific survival; RFS: Recurrence-free survival; OS: Overall survival.

no lymphadenectomy (10.9%, 10/91) groups (P = 0.03; Figure 5). We should emphasize that 75% (3/4) of regional node recurrences in the incomplete lymphadenectomy group were found outside the dissected sites. Complete lymphadenectomy was a predictive factor for a reduced risk of regional node recurrence. Thus, this study shows the role of template-based lymphadenectomy in reducing the risk of regional node recurrence in renal pelvic cancer, which may in turn be associated with improved patient survival. At the same time, our study suggests that the prevention of regional node recurrence by lymphadenectomy is attributed to the dissection of tumor microdeposits in the regional lymph nodes.

Abe *et al*[30] confirmed the above hypothesis by examining micrometastases to the lymph nodes using immunohistochemistry with an anticytokeratin antibody. They demonstrated that 14% of patients with no metastases, as diagnosed by regular hematoxylin-eosin staining, showed a positive immunohistochemical reaction for micrometastases. In addition, the majority of patients with micrometastases survived for a long time after lymphadenectomy.

We also examined lymph node micrometastases by examining the expression of urothelial carcinoma-specific markers in lymphadenectomy specimens using the quantitative reverse transcription-polymerase chain reaction[40]. We found that this technique detected micrometastases in about 10% of patients who had no metastases according to routine hematoxylin-eosin staining. Moreover, the prognosis of the patients was stratified well according to the metastatic status of the lymph nodes.

Collectively, these results show that the therapeutic benefit of lymphadenectomy is likely to be attributed to the surgical resection of microtumor deposits that spread to the lymph nodes in UCUT. Again, we should emphasize that the therapeutic benefit could not be obtained without anatomical template-based lymphadenectomy.

UNDERLYING ISSUES REGARDING LYMPHADENECTOMY

Minimum number of lymph nodes removed that influence patient survival

The number of lymph nodes removed is likely to be a good indicator for assessing the extent of lympha-

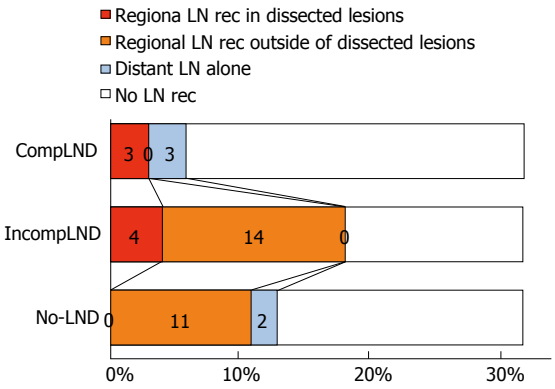

Figure 5 Recurrence pattern in regional nodes according to the extent of lymphadenectomy in patients with renal pelvic cancer. LND: Lymphadenectomy; LN: Lymph node.

denectomy in bladder cancer[41-43]. Reportedly, survival rates continued to rise as the number of resected lymph nodes increased[44]. The question is whether a minimum number of lymph nodes should be resected to influence patient survival in UCUT.

Roscigno *et al*[28] reported that a minimum of 7 lymph nodes should be removed to significantly improve survival in patients with pT2 stage tumors or higher. A multi-institutional study showed that the removal of 8 lymph nodes or more resulted in higher CSS compared to the removal of less than 8 lymph nodes in patients with ≥ pT1pN0[37]. In this patient cohort, the risk of cancer mortality continued to decrease as the number of lymph nodes removed increased, as in bladder cancer.

We also examined whether there is a minimum number of resected lymph nodes that can affect patient survival[45]. Our results showed that there was no cutoff value that significantly influenced patient survival (Figure 6). Eight lymph nodes were likely to be a minimum requirement for improving patient survival, but it was not a statistically significant value. In contrast, template-based lymphadenectomy was significantly associated with a higher CSS rather than incomplete lymphadenectomy where all regional sites were not resected. Thus, lymphadenectomy should be performed by following the anatomical template. We believe that the number of resected lymph nodes cannot be used to determine the extent of lymphadenectomy, but can be used for assessing the adequacy of lymphadenectomy.

Indication of lymphadenectomy

It is important to determine the indication of lymphadenectomy in UCUT. Patients who benefit from lymphadenectomy have been examined. According to studies that analyzed the benefit of lymphadenectomy (Table 2), this role is limited in patients with pT2 stage tumors or higher. The therapeutic benefit of lymphadenectomy is also more clearly demonstrated in patients with pT2 stage tumors or higher (Table 3). Thus, an indication for lymphadenectomy is assumed in patients with pT2 tumors or higher. This is also supported by the results showing the incidence of lymphatic metastases according to pathological stage. Our data shows that the incidence of lymph node metastases increases incrementally as the pathological stage becomes higher (Figure 7). The risk of lymphatic metastases was only at 1% in the patients with pT1 stage tumors or lower, whereas tumors at the pT2 stage show a 7% risk of lymph node metastases. This risk increases to 26% in pT3 tumors. It is reasonable to perform lymphadenectomy in patients with pT2 tumors or higher.

However, there is a major concern about accurate preoperative staging based on current radiological modalities. Although multidetector computed tomography might provide more accurate staging[46], it is likely to be very difficult to distinguish stage 1 from 2. In other words, invasion of the muscle layer of the renal pelvis or the ureter is very difficult to diagnose according to our results[47]. Some tumors clinically diagnosed as carcinoma in situ may upstage to the muscle-invasive diseases pathologically[48]. Thus, we currently consider all patients with an indication of nephroureterectomy as candidates for lymphadenectomy. We omit lymphadenectomy for patients of an advanced age or with severe comorbidity[47].

Association with neoadjuvant or adjuvant chemotherapy

The role of neoadjuvant therapy has been discussed recently since a majority of patients is unfit for cisplatin-based chemotherapy after nephroureterectomy because of the development of chronic kidney disease[49,50]. In addition, adjuvant chemotherapy has little effect on improving survival[51,52]. Thus, the role of neoadjuvant chemotherapy has recently attracted physicians' attention.

A recent retrospective study showed that cisplatin-based neoadjuvant chemotherapy significantly improved patient survival[53]. In addition, multivariate analysis showed that lymphadenectomies where more than 8 lymph nodes were resected were no longer a significant factor when neoadjuvant chemotherapy was included. Furthermore, it is difficult to draw a definitive conclusion from these results, which are from a single institute. However, further study is warranted to elucidate the association between the benefit of lymphadenectomy and neoadjuvant chemotherapy.

Adjuvant chemotherapy might enhance the therapeutic benefit of lymphadenectomy. Several studies examined the effect of adjuvant chemotherapy, but most failed to show an improvement in patient survival[51,52,54-56]. We examined the role of adjuvant chemotherapy in a retrospective study. Lymphadenectomy was a significant independent factor reducing the risk of cancer mortality, but adjuvant chemotherapy was not a significant factor, even in the univariate analysis (HR = 1.89, 95%CI: 0.677-5.43; $P = 0.222$)[35]. Our prospective study also showed that adjuvant chemotherapy does not influence either cancer-specific or disease-free survival on univariate analysis in patients with renal pelvic cancer[23]. Thus, these results suggest that the therapeutic benefit of lymphadenectomy

CSS according to the total No. of LNs removed in ≥ pT2

	NO. LNs	Cut-off value						
		5	6	7	8	9	10	11
5y CSS	< Cut-off	64.5	60.7	60.4	64.4	67.4	70.1	70.1
	≥ Cut-off	76.4	82.2	87.3	86.7	83.6	78.0	78.0
P-value		0.64	0.17	0.10	0.07	0.22	0.62	0.62

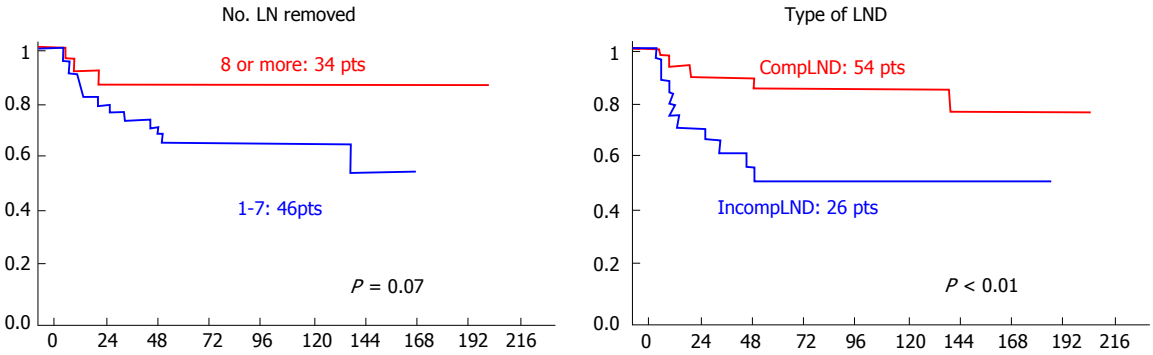

Figure 6 The influence of the number of lymph nodes removed on cancer-specific survival. LND: Lymphadenectomy; CSS: Cancer-specific survival; LNs: Lymph nodes.

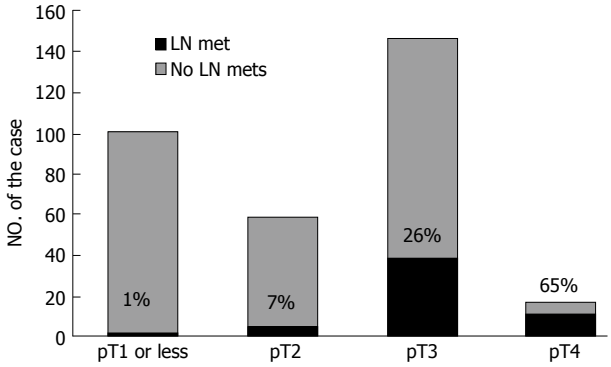

Figure 7 The incidence of lymphatic metastases according to the primary tumor stage. LN: Lymph node.

is independent, but not synergistic with adjuvant chemotherapy.

Is laparoscopic or robotic lymphadenectomy feasible?

Lymphadenectomy was performed using an open procedure for all the patients in our study. The median yield of lymph nodes from template-based lymphadenectomy was 15 in renal pelvic cancer and 14 in ureteral cancer for our prospective study[23]. This number is believed to be the current standard, but it was only 7 in the lymphadenectomy cohort before 2006[35].

Laparoscopic lymphadenectomy results were reported by Abe *et al*[57] showing that the median number of resected lymph nodes was 10. They recently reported the prospective results for their current laparoscopic lymphadenectomy procedure[58]. The median number of lymph nodes removed increased to 14, which is very similar to the number from our prospective study for open lymphadenectomy[23]. Thus, experienced surgeons can perform laparoscopic lymphadenectomy as effectively as an open procedure. However, a long learning curve will be required.

Very few results of robotic lymphadenectomy with nephroureterectomy have been reported. Pugh *et al*[59] reported that the median number of lymph nodes was 11. The mean number of resected lymph nodes was 14.1 according to Lee *et al*[60]. These results are very similar to those from our prospective study. Our opinion is that robotic lymphadenectomy may be feasible. The appropriate procedure can be determined by the surgeons' experience. However, we believe that an open procedure is the most reliable and the experience of surgeons is not likely to influence its quality.

What are the disadvantages of lymphadenectomy?

The disadvantages of lymphadenectomy in UCUT should also be considered. In our prospective study, we compared the incidence of complications in the template-based lymphadenectomy group to that of the no lymphadenectomy group (Table 4)[23]. Patients who undergo template-based lymphadenectomy show a higher incidence of complications at all grades as well as grade 3 or higher, but without a significant difference. More frequent complications in the lymphadenectomy group include numbness in the thighs and lymphorrhea. Lymphorrhea including chyle fistulas occur at a higher incidence and grade in those who undergo lymphadenectomy than those who do not (5.2% *vs* 1.1%). One patient required percutaneous drainage, but conservative management spontaneously resolved the problem in other patients. Numbness in the thigh may be associated with lymphadenectomy for pelvic nodes (2.5% *vs* 0%).

Rao *et al*[38] reported complications from a prospective study of super-extended lymphadenectomy that encompassed the area from the retroperitoneum to the pelvis. The morbidities in this study were transfusion (32%), ileus (5%), and chylous leakage (10%). Chylous leakage was managed with conservative treatment except for 1 patient for whom surgical intervention was

Table 4 Perioperative complications of the template-based lymphadenectomy and the no lymphadenectomy group

Template-based lymphadenectomy (77 patients)			No lymphadenectomy (89 patients)	
	Morbidity	*n*	Morbidity	*n*
	Grade 1		Grade 1	
	Numbness of thigh	2	Atelectasis	1
	lymphorrhea	1	Delirium	2
	Wound infection	1	Wound infection	2
	Grade 2		Lymphorrhea	1
	Chylous leakage	1	Subcutaneous hematoma	1
	Retroperitoneal abscess	1	Grade 2	
	Lymphorrhea	1	Anemia	1
	Gastric ulcer	1	Grade 4	
	Grade 3a		Intraoperative massive bleeding	1
	Lymphorrhea	1		
	Grade 3b			
	Rectal injury	1		
	Ureteral injury	1		
Incidence (all grades)		11		9
		14.20%		10.10%
Incidence (≥ grade 3)		3		1
		3.90%		1.10%

needed.

We also compared intraoperative bleeding and operation time in patients who underwent template-based lymphadenectomy or no lymphadenectomy. The lymphadenectomy group showed more intraoperative bleeding and longer operation times (407 mL *vs* 321 mL, 323 min *vs* 288 min), but there was no significant difference[19]. The length of hospital stay after surgery did not differ between groups. A randomized prospective study examining the role of lymphadenectomy in renal cell carcinoma showed no increase in complications from extensive lymphadenectomy compared to no lymphadenectomy (26% *vs* 22%)[61].

We performed lymphadenectomy in an open procedure with a retroperitoneal approach in all patients. Thus, we cannot comment on transperitoneal lymphadenectomy for UTUC. However, in the above randomized phase 3 trial for kidney cancer, all surgeries were done with a transperitoneal open procedure[61]. Thus, we believe that lymphadenectomy does not increase the risk of complications, irrespective of the approach used.

Thus, lymphadenectomy may result in a slight increase in complications including lymphorrhea or hemorrhage, but has no influence on patients' recovery from surgery. We should consider the complications of lymphadenectomy; however, they should not dissuade surgeons from performing lymphadenectomy except in patients with comorbidity or of an advanced age.

CURRENT RECOMMENDATIONS FOR LYMPHADENECTOMY IN THE 2015 GUIDELINES

Four guidelines are currently available for UCUT. The latest European Association of Urology guidelines (2015 version) recommend lymphadenectomy for cases of invasive disease[62]. The recommendation grade is still low at grade C. The National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology Version 1.2015 state that lymphadenectomy should be a part of nephroureterectomy for high-grade tumors, or tumors that are large and invade the renal parenchyma[63]. The National Cancer Institute-Physician Data Query suggests that lymphadenectomy at the time of radical nephroureterectomy may offer prognostic information, but little, if any, therapeutic benefit[64]. The guideline of the Japanese Urological Association also supports the staging benefit, and recommends lymphadenectomy to improve survival in patients with advanced disease with suspected muscle invasion as a grade C recommendation[65].

Thus, the current recommendation grade for lymphadenectomy still remains low; however, our nonrandomized prospective study is not incorporated[23]. The role of lymphadenectomy is expected to be supported by guidelines at a higher level than at present, especially in renal pelvic cancer.

CONCLUSION

Herein, the current situation and issues of lymphadenectomy for UCUT have been summarized. There are some major problems underlying lymphadenectomy, including the lack of standardization of the extent of lymphadenectomy and a randomized prospective trial. However, we believe that lymphadenectomy is strongly recommended for tumors of the renal pelvis. Lymphadenectomy should follow the anatomical template. Further research is warranted to establish the role of lymphadenectomy in UCUT.

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REVIEW

Biomarkers in triple negative breast cancer: A review

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Abstract

Breast cancer is an intrinsically heterogeneous disease. In the world about 1 million cases of breast cancer are diagnosed annually and more than 170000 are triple-negative. Characteristic feature of triple negative breast cancer (TNBC) is that it lacks expression of oestrogen, progesterone and human epidermal growth factor receptor-2/neu receptors. They comprise 15%-20% of all breast cancers. We did a systematic review of PubMed and conference databases to identify studies published on biomarkers in TNBC. We included studies with biomarkers including: Epidermal growth factor receptor, vascular endothelial growth factor, c-Myc, C-kit and basal cytokeratins, Poly(ADP-ribose) polymerase-1, p53, tyrosinase kinases, m-TOR, heat and shock proteins and *TOP-2A* in TNBC. We also looked for studies published on synthetic lethality and inhibition of angiogenesis, growth, and survival pathways. TNBC is a complex disease subtype with many subclasses. Majority TNBC have a basal-like molecular phenotype by gene expression profiling. Their clinical and pathologic features overlap with hereditary *BRCA1* related breast cancers. Management of these tumours is a challenge to the clinician because of its aggressive behaviour, poor outcome, and absence of targeted therapies. As the complexity of this disease is being simplified over time new targets are also being discovered for the treatment of this disease. There are many biomarkers in TNBC being used in clinical practice. Biomarkers may be useful as prognostic or predictive indicators as well as suggest possible targets for novel therapies. Many targeted agents are being studied for treatment of TNBC.

Key words: Triple negative breast cancer; Epidermal growth factor receptor; Vascular endothelial growth factor; p53; Cyclin

Core tip: Triple negative breast cancer (TNBC) are type of breast cancer which lack of estrogen receptors, progesterone receptors and human epidermal growth factor receptor. It is a complex disease subtype with many subclasses. There are many biomarkers in TNBC used for its sub-classification. Clinically-practical assay/biomarkers that can reliably identify TNBC are

necessary. Biomarkers may be useful as prognostic or predictive indicators as well as suggest possible targets for novel therapies.

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INTRODUCTION

Breast cancer is a complex disease entity with different biological characteristics and clinical behaviour. Many clinical and pathological features have been defined to predict outcome and treatment response in breast cancer. These features include: Patient age, tumour stage, axillary lymphnode involvement, lymphovascular invasion, histologic grade, hormonal and human epidermal growth factor receptor (HER-2/neu receptor) status. In the past chemotherapy was the only systemic therapy for triple negative breast cancer (TNBC) patients. Currently lot of research is going on to further characterise TNBC with different molecular markers and find targets for therapy in order to improve its outcome. Sørlie *et al*[1] has diversified five subgroups of breast cancer by gene expression profiling (GEP) using DNA microarrays. These are luminal A, luminal B, HER-2/neu over expressing, basal like (BL) and normal like breast cancer. BL breast cancer lacks estrogen receptors (ER), progesterone receptors (PR) and HER-2/neu receptors, thus contribute to 80% of TNBC[1,2] The present review provides an insight into the different biomarkers in TNBC and its sub classification based upon the marker profile to understand molecular targets in each subtype.

TNBC

TNBC[3] are type of breast cancer which lack ER, PR and HER-2/neu receptors. It has different and poor clinical and pathological features as compared to other subtypes of breast cancer. It is usually seen in young age, advanced stage at presentation, unfavourable histopathology, grade Ⅲ, higher proliferative index, lack of tubule formation and higher rate of metastases[4-9]. It is associated with higher rate of local recurrence during 3 year after treatment and a high 5 year death rate[10]. Survival is poor after distant metastasis[11,12]. TNBC frequently affects younger patients (< 50 years) and has higher prevalence in the African-American women[13]. Patients with TNBC has inferior disease free survival (DFS) and overall survival (OS) as compared to age and grade matched controls of non-TNBC patients[11]. In TNBC metastatic rate is high to visceral organs[14,15] and lung and cerebral metastasis is more common[16-19]. Cytotoxic chemotherapy is the only treatment option[20-22].

TNBC subtypes

TNBC is a distinct breast cancer. It is classified into six groups based upon the GEP and DNA microarray. This sub-classification is not only useful in understanding the disease better but also to find molecular targets for its treatment[23].

BL-1 and BL-2: The BL-1 subtype was found to be composed rapidly dividing cells associated with increased proliferation and cell cycle checkpoint loss consistent with the increased expression of DNA damage response genes. Due to its high proliferation rate it has increased Ki67 mRNA expression and it is more responsiveness to antimitotic agents targeting cell cycle. The BL-2 subtype on the other hand displayed unique gene ontologies involving epidermal growth factor signalling as well as glycolysis and gluconeogenesis pathway. On microarray it showed a higher expression of epidermal growth factor receptor (EGFR), TP63, MET, *etc*.

Immunomodulatory subtype: Immunomodulatory (IM) is composed of immune cell responses such as immune cell and cytokine signalling, antigen presentation and processing and signalling of immune transduction pathways. Its GEP substantially overlaps with the medullary breast cancer, histologically a rare distinct form of TNBC which carry favourable prognosis despite its high grade.

Mesenchymal and mesenchymal stem like subtype: On GEP these subtypes consists of epithelial-mesenchymal (M) transition and growth factor pathways. The mesenchymal stem like subtype is also expressed by genes involved in angiogenesis including VEGFR2 and was found to be highly responsive to dasatinib [tyrosine kinase (TK) inhibitor], and mTOR inhibitors.

Luminal androgen receptor subtype: This subtype is characterised by androgen receptor (AR) signalling. It is ER negative but gene ontologies were heavily composed of hormonally regulated pathways such as steroid synthesis, porphyrin metabolism and androgen/estrogen metabolism. AR mRNA expression was nine times higher than other subtypes therefore, these lines were found to be highly sensitive to AR antagonists eg biclutamide. Patients with this subtype had decreased DFS and OS.

Basal cell and TNBC

Among TNBCs 80%-90% falls into the category of BL molecular subtype when appropriately tested for IHC cancer biomarkers and GEP but these terms are nonsynonymous and are overlapping[10,24]. At present, there is no optimal IHC panel for identification of basal like breast cancer (BLBC). Therefore TNBC, despite having above limitations is considered as a BL cancer. In a study Thike *et al*[9] with a tri-panel of cytokeratin-14 (CK-14) , EGFR and 34βE12 in TNBC reported 84% to be BL tumors with a specificity and sensitivity of 100% and 78% respectively. In BLBC over expression of ID4 leads to the deregulation of *BRCA1.* BLBCs are also

Table 1 Epidermal growth factor receptor expression in triple negative breast cancer

Ref.	Total number	No. of TNBC subjects	EGFR expression[1]
Thike *et al*[9], 2010	7048	767	30%
Patil *et al*[10], 2011	683	136	7.4%
Nielsen *et al*[24], 2004	-	21 basal like tumours	57%
Rakha *et al*[45], 2007	1726	282	37% in TNBC *vs* 15% in non-TNBC
Mehdizadeh *et al*[47], 2012	1132	103	23.3%
Rydén *et al*[48], 2010	564	48	41% TNBC *vs* 11% non-TNBC

[1]The expression is depicted as the percentage of patients expressing the marker. TNBC: Triple negative breast cancer; EGFR: Epidermal growth factor receptor.

known to have either p53 over expression or mutations in the gene[24].

In array, BLBCs are characterised by low expression of ER and HER-2 related genes, so pathologically they are usually ER-negative, PR-negative and lack HER-2 over expression[8,9] or are < 1%; < 5%; 10%; 20% immunoreactive for the above receptors[24]. They stains positive for cytokeratins (CKs) 5/6 and 17, and over express EGFR (HER1). Furthermore they show a highly aggressive GEP with low Bcl-2 but high p53 and Ki67[25-29].

BRCA AND TNBC

Genetic instability leads to cancer predisposition. Genetic mutations in the *BRCA* genes in patients predisposes them to develop many cancers such as breast, ovarian, pancreatic and prostate. *BRCA 1* plays vital role in DNA repair by homologous recombination. Inactivation of this gene due to *BRCA* mutation should trigger cell cycle arrest but this too is inhibited by p53 mutations in TNBC[30]. Lack of a functional *BRCA1/2* in cells lead to loss of repair of DNA double-strand breaks (DSB). This mechanism leads to increased risk of cancer in these patients. Histologically and transcriptionally, TNBC share similarities with *BRCA1*-linked breast cancers, which means that dysfunction of *BRCA1* is seen in TNBCs[31,32]. TNBCs are heterogeneous with respect to GEP. TNBC is associated with cancers arising in *BRCA1* mutation carrier in young women as compared to those in their late forties. Both sporadic BLBCs and *BRCA1* associated breast cancers have evidence of genomic instability. More than 80% of breast cancers in women who carry germ-line *BRCA1* mutations are TN and 10% TN breast tumors have *BRCA1* mutation. The reasons for these associations are unclear but may ultimately provide avenues for prevention as well as targeted therapy with poly(ADP-ribose) polymerase (PARP) inhibitors and chemotherapy with DNA-damaging agents such as platinum compounds[33-35].

Biomarkers in TNBC

TNBC is characterised by the marked expression of certain biomarkers. The presence of these molecules though is not restricted to TNBC but somehow show increased prevalence in this subgroup. The following are the important biomarkers in TNBC.

EGFR: EGFR is one of the members of four closely related receptors each playing an important role in tumour cell survival. The four receptors being EGFR (or ErbB-1), HER-2/neu (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4)[36,37]. The inactive monomer receptor dimerizes after ligand activation followed by TK, intracellular domain of the receptor is activated by autophosphorylation, leading to cascade of intracellular events. EGFR signal cascade is important for cell proliferation, angiogenesis, metastatic spread, and the inhibition of apoptosis[38]. Most of the TNBCs express EGFR, and poses a strong therapeutic challenge[39]. Studies with different methods of gene amplification have found variable expression EGFR in metaplastic breast carcinoma, a phenotypes of BLBCs[40-42]. However, Toyama *et al*[43] with real-time polymerase chain reaction have reported high *EGFR* gene copy number in TNBCs. EGFR expression is found in 40%-50% of patients with breast cancer and in 80% of TNBC; and is estimated to substitute major proliferation pathways of breast cancer induced by activation of HER-2, ER, PR proteins which are thereby absent in TNBC[25].

In a study the authors found that 60% of patients with grade Ⅲ and > 3 lymph nodes showed EGFR expression, indicating that EGFR expression is related to aggressiveness of the disease. They also concluded that patients with EGFR expression had worse DFS, distant disease free survival (DDFS), OS and cause specific survival[44]. EGFR expression in TNBC is associated with poor response to chemotherapy[45]. Nogi *et al*[46] observed that EGFR was expressed in 24% of the TNBC patients and was related to less favourable response to chemotherapy and poorer survival and on the contrary the luminal groups where EGFR expression showed good response to chemotherapy and better survival. Recently EGFR has been defined with other markers to differentiate BL subtype from TNBC[47]. This aids in segregating TNBC into subtypes and thus defining the prognostic difference and molecular target specification between the two. Non-uniformity of expression profiles in studies shown in Table 1 is due to absence of subtype consideration or BL subtype non segregation from core TNBC. So EGFR is a biomarker in TNBC and a target for cetuximab, a TK inhibitor[48]. Many studies have evaluated its response in TNBC[48-51]. In a recent study, EGFR expression was shown as prognostic factor for DFS

Table 2 Vascular endothelial growth factor receptor expression in triple negative breast cancer

Ref.	Total number	No. of TNBC	VEGFR-2 expression[1]
Mehdizadeh *et al*[47], 2012	1132	103	93.2%
Iosifidou *et al*[62], 2009	-	73	77%
Chanana *et al*[63], 2012	70	27	54% *vs* 23%
Linderholm *et al*[67], 2008	679	87	Higher intratumour VEGF levels in TNBC
Andre *et al*[68], 2009	69	35	34%

[1]The expression is depicted as the percentage of patients expressing the marker. TNBC: Triple negative breast cancer; VEGFR: Vascular endothelial growth factor receptor; VEGF: Vascular endothelial growth factor.

not only in univariate but also in multivariate analysis[52].

Vascular endothelial growth factor: Angiogenesis is important for tumour growth and spread especially beyond a diameter of 2 mm as oxygen and nutrients cannot diffuse beyond this distance. Angiogenic signals are mediated by vascular endothelial growth factor (VEGF) to aid neovascularisation. VEGF A, B, C, D, E (viral factor) and placental growth factor is a family of six proteins. VEGF protein is found in 4 isoforms because of alternative splicing of its mRNA[53,54]. Among the different isoforms VEGF165, the 165-amino acid molecule is more common[55,56]. Its gene expression is controlled by many of stimuli such as hypoxia, nitric oxide, growth factors, oncogenes, tumour suppressor genes and HER-2[57].

It causes proliferation and maintains structural and functional integrity of cells of the endothelium. It also regulates vascular permeability and migration of endothelial stem cells from the bone marrow[58]. Neovascularisation in the tumour is also regulated by VEGF by increasing the expression of the anti-apoptotic proteins such as Bcl2, XIAP, and survivin. In its absence the endothelial cells undergo apoptosis and newly formed vessels disintegrate[59-61]. Thus neovascularisation is dependent on VEGF expression throughout tumour development. VEGF shows multiple interactions with receptor TKs, such as VEGFR-1, VEGFR-2, and VEGFR-3. The angiogenesis is initiated by VEGF binding to VEGFR-2 which triggers the specific activation of TKs followed by multiple signalling cascades resulting in the endothelial cells survival, proliferation, migration, adhesion, actin remodelling and vessels permeability[62].

VEGF expression is elevated in DCIS and invasive breast cancer. It has been also well utilised for prognosis in breast cancer[63,64]. Its quantification by IHC or immunoassay of tissue extracts has shown a significant co relation with micro vessels counts or density. High mean vascular density in breast cancer has been found to linked with more aggressive tumour behaviour and poor survival so intratumoral microvessels density is now considered as one of the important factors affecting survival[65]. According to recent studies[63,66] there was a direct co relation between serum and tissue levels of VEGF to grade Ⅲ tumours, larger tumour size, positive lymph node and negative hormone status and poor survival along with a substantial decrease in levels with chemotherapy. In TNBC higher VEGF levels are associated with shorter DFS, OS, and DDFS. Also VEGF levels have been significantly related to size of the tumour, grade and metastatic sites. In patients with higher VEGF levels disease progressed despite of therapy and such patients were associated with significantly lower progression free survival as compared to patients with lower levels. In TNBC patients it was found that VEGF level elevated from baseline to middle of the therapy significantly but showed a non significant increase from middle of the therapy to its end when patients were administered FAC[65-67]. VEGF is a target for bevacizumab in TNBC patients. Table 2 shows VEGF expression reported in different studies.

C-kit and basal cytokeratins: C-kit is a cytokine receptor present on the surface of hematopoietic stem cells and also in other cells. C-kit binds to stem cell factor and is a growth factor receptor that stimulates major cellular functions such as cell survival, proliferation, differentiation, adhesion and chemotaxis. It induces apoptosis and also increases the invasiveness of the cancer cells[68]. CKs are keratin-containing proteins of intermediate filaments found in the intracytoplasmic cytoskeleton of epithelial tissue. Different epithelial tissues express different CKs at the time of its terminal differentiation and the stage of development. This different CK expression helps in the classification of all epithelia. Similarly different cancers express specific CKs of that epithelium. Therefore the CK expression profile tends to remain constant when an epithelium undergoes malignant transformation.

The study of the CK profile by IHC techniques is very important for tumor pathologic classification[69]. These CKs were earlier used to distinguish malignant breast lesions from benign ones[70], but later their prognostic value was ascertained and it was seen that expression of CK-5, CK-14 and CK-17 was related to poor prognosis, high grade tumours, ER negativity, short DFS and OS[71-73]. It is expressed in BLBCs. Since BLBC and TNBC show overlapping features therefore C-kit and basal CKs along with other markers and pathological features are used for the differentiating BLBCs from

Table 3 C-kit expression in triple negative breast cancer

Ref.	Total number	No. of TNBC	C-kit expression[1]
Thike *et al*[9], 2010	7048	767	CK 5/6 in 6%, CK-14 in 48%, CK-17 in 50%, C-kit in 45%
Nielsen *et al*[24], 2004	-	21	CK 5/6 in 62% and C-kit in 29%
Kim *et al*[76], 2009	625	147	CK5/6 in 35.4% and C-kit in 11.6%
Bryan *et al*[78], 2006	66	4	75% of TNBC *vs* 29% of non-TNBC

[1]The expression is depicted as the percentage of patients expressing the marker. TNBC: Triple negative breast cancer; CK: Cytokeratin; EGFR: Epidermal growth factor receptor.

TNBC. Many studies have revealed that presence of CKs is higher in TNBC than non-TNBC and also among TNBC subgroup it is higher in the BL subclass (Table 3). BL subclass of TNBC was identified on the basis of CK and EGFR expression and when the clinicopathological features were compared between the basal and non-BL it was seen that BL subclass of TNBC were more aggressive[9,74-78].

p53: It is a tumour suppressor protein which is encoded by the *TP53* gene (the tumour suppressor gene). It is also called the "guardian of genome" as it is important cell cycle regulator[79]. It regulates cell growth, multiplication, proliferation and apoptosis, and promotes chromosomal stability. Disruption of these functions by mutation in the gene producing p53 lead to carcinogenesis. p53 is activated in response to cellular stress by many pathways that are dependent on distinct upstream regulatory kinases. First, an ataxia-telangectasia mutated proteins released in response to the DSB, second, a pathway dependent on *INK4* gene product, p14ARF activated by oncogenes, and finally, a pathway induced by chemotherapy drugs and ultraviolet light and is independent of the above two pathways[80,81].

p53 mutations are seen in 18%-25% of primary breast carcinomas (Table 4)[82]. p53 plays an important role in breast cancer prognosis. p53 over expression leads to poor response to chemotherapy[83,84]. Many studies have reported that its activation is associated with aggressive form of breast cancer and significantly decreases DFS and OS in TNBC patients[85-88]. Also co existence with HER-2 was significantly related to early relapse and death within shorter period after surgery[87]. Along with EGFR and cytokeratins it is used for segregation of a subclass, *i.e.*, basal like from core TNBC[89].

Tumours with p53 mutation are highly invasive, poorly differentiated and high grade tumours. In a study by Chae *et al*[90], p53 mutation was associated with poor response to the chemotherapy in TNBC patients. Other proteins of p53 family are p63/p73 proteins. Tumors expressing these proteins are reported to have many folds higher sensitivity to platinum based chemotherapy. p63/p73 expression is seen in one-third of patients with TNBC[91].

***TOP-2A*:** This gene encodes topoisomerase Ⅱ α and plays a crucial role in DNA transcription. This enzyme causes the temporary break of double strands of duplex DNA and rejoins them so that the strands cross through one another, therefore altering the topology of DNA. Mutation in cancer leads to depreviation of its functions and thus worsening of the situation. In TNBC or breast carcinoma the gene acts as a target for anthracycline therapy which is a topoisomerase Ⅱ inhibitor[92]. So it is a marker for the evaluation of resistance to the anthracycline therapy. A study revealed a higher expression of *TOP-2A* in 2.7% to 8.8% of TNBC patients[93]. Its over expression in TNBC leads to the decreased sensitivity towards the anthracyclines and thus decreased response[94].

Ki67: Also known as MKI67, Ki67 is a cellular marker for proliferation. Ki67 antigen is present inside the cell nucleus during interphase and during mitosis it is relocated to the surface of the chromosomes. Since it is a marker of proliferation it is found in all cells when they are in dividing phases of the cell cycle (G_1, S, G_2, and mitosis) and it is absent from cells during their resting phase (G_0). Its absence in resting cells and generalised presence in dividing cells had made it a marker of cell proliferation[95]. Proliferation is a salient feature for the spread of cancer and can be assessed by the IHC measurement of the nuclear antigen Ki67. It's over expression also correlates with levels of bromodeoxyuridine uptake and S-phase fraction, other markers of proliferation.

Ki67 expression is less in normal breast tissue (< 3%). It has been reported in many studies that Ki67 antigen and steroid-receptor are expressed in different cells in normal human breast epithelium. Ki67 was over expressed particularly in ER-negative cells and its expression in carcinoma cells was much higher[96,97]. In breast cancer high Ki67 is associated with of poor outcome although these tumours show very good clinical response to combination chemotherapy. However, its independent significance is modest and does not merit measurements in routine clinical practice. With respect to treatment response in breast cancer, Ki67 expression was found to be independent predictor of pathologic complete response (pCR), clinical complete response, OS and DDFS and locoregional recurrence. It was also seen that patients without pCR still showed a decrease in Ki67 index post therapy[98-100]. In a recent meta-analysis

Table 4 p-53 expression in triple negative breast cancer

Ref.	Total number	No. of TNBC	p-53 expression
Patil *et al*[10], 2011	683	135/683	47.8%
Nielsen *et al*[24], 2004	11	11	82%
Rakha *et al*[45], 2007	1726	282/1726	56% in TNBC *vs* 22% in non-TNBC
Chae *et al*[90], 2008	135	32/135	40.6% in TNBC *vs* 42.7% in non- TNBC
Biganzoli *et al*[89], 2011	-	(633 + 1026) from two separate sources	Divided TNBC into subclass BL which accounts for 89% of total TNBCs

TNBC: Triple negative breast cancer; BL: Basal like.

by de Azambuja *et al*[101] who retrieved DFS data from 29 studies, they concluded that high Ki67 levels was associated with poor prognosis in irrespective of nodal status and whether patients undergo treatment or not at all.

In TNBC, it was found that Ki67 levels were significantly increased in ductal TNBC compared to other histologic types (80% in TNBC *vs* 10%-30% in other types). Its expression also represented a direct co relation with tumour size and grade in TNBC patients and higher levels (> 35% staining) were linked with an increased risk of death[102,103]. In TNBC patients Ki67 accumulation was associated with a higher pCR to chemotherapy but poor RFS and OS. Its expression was also used for subdivision of TNBC into two subtypes where only 26.7% of TNBC patients showed lower Ki67 expression[104].

***PARP*:** *PARPs* are a family of cell signalling enzymes present in eukaryotes, which catalyses the poly(ADP-ribosylation) of DNA binding proteins. Till now eighteen enzymes of *PARPs* has been detected, but *PARP1* the most common isoform. *PARP1* is responsible for majority of its functions. Main function of *PARP1* is as DNA damage nick sensor. It forms polymers of ADP-ribose and nicotinamide with use of NAD^+. Activation of *PARP1* is important in tumours because of three interesting biological reasons: First, it plays a vital role in DNA repair through base excision repair pathway; second, it is capable of depleting cellular energetic pools, which results in cell dysfunction and necrosis; and third, its ability to promote the transcription of proinflammatory genes. *PARP* enzymes are involved in cellular response in inflammation, ischemia and oxidative stress. Carcinogenesis is a multistep process involving alterations in many cellular processes such as genomic stability, cell division, proliferation, growth, differentiation and cell death. *PARP1* are involved in all these cellular processes, indicating possible link between *PARP1* function and carcinognesis[105]. *PARP1* repairs DNA single strand breaks (SSB) by binding to the exposed ends of the damaged DNA strand and bring in important enzymes required for repair in SSBs[106-110]. The base excision repair pathway fails when *PARP1* is inhibited; this leads to accumulation of SSBs. In a dividing cell entering S-phase, cell division is arrested at SSBs, leading to a DSB (Figure 1). In *BRCA1* deficient cells excision repair pathway is dependent on *PARP1*, inhibition of *PARP1* leads to cell death through apoptosis[106,107]. *BRCA2* operates through excision repair pathway like *BRCA1*, mutation of this gene make the cells suceptible to *PARP* inhibitors as well[109,110]. *PARP* also plays a vital role in DNA repair as *BRCA.* Unlike *BRCA* it recognises SSBs and repairs by base excision repair pathway[105]. *PARP* inhibitors are effective in TNBC because damage to one of the arms of the DNA could not be repaired by homologous recombination due to *BRCA* mutation and *PARP* inhibition in synergism will create a state of "synthetic lethality" - a process that occurs when inactivation of individual genes have no effect but mutations in both the genes lead to death of cancer cells[107]. So *BRCA* mutation is responsible for the action of many chemotherapeutic agents in TNBC. The inhibition of *PARP1* is also known to potentiate the effect of ionizing radiation and many drugs such as DNA methylating agents, topoisomerase I inhibitors, and platinum compounds. Studies in mouse models have shown that the addition of *PARP* inhibitors with platinum compounds increases RFS and OS[35,105,107] while many of other studies on cell lines reveal that the activity of *PARP* inhibitors was increased in presence of *BRCA* mutations or dysfunction[105,108]. *PARP1* has been targeted as therapeutic option in TNBC with drugs like iniparib, olaparib etc though not found to be independently helpful but their addition to cytotoxic agents have surely brought synergism to their activity and improvement in treatment response in TNBC patients.

Heat shock protein 90: It is a cellular chaperone (proteins that assist the assembly or disassembly of other macromolecular structures) protein that mediates the post-translational modification and stabilization of a number of conformationally labile proteins, steroid receptors, cyclin-dependent kinase 4, RAF-1, AKT and other proteins that are useful for sending proliferative signals[111]. Once function of heat shock protein (HSP) 90 is blocked, its dependent proteins are broken by proteosomes. Small HSP αB-crystalline is expressed in BLBCs and is associated with shorter survival. Its' over expression is associated with neoplastic changes in mammary acini, increases cell migration and invasion in vitro. Geldanamicyn and tanespimycin both are antibiotics and inhibitors of HSP. These have shown clinical benefit in HER2-positive metastatic breast cancer[112]. The PU-H71 another HSP blocker has shown complete response in TNBC models[113].

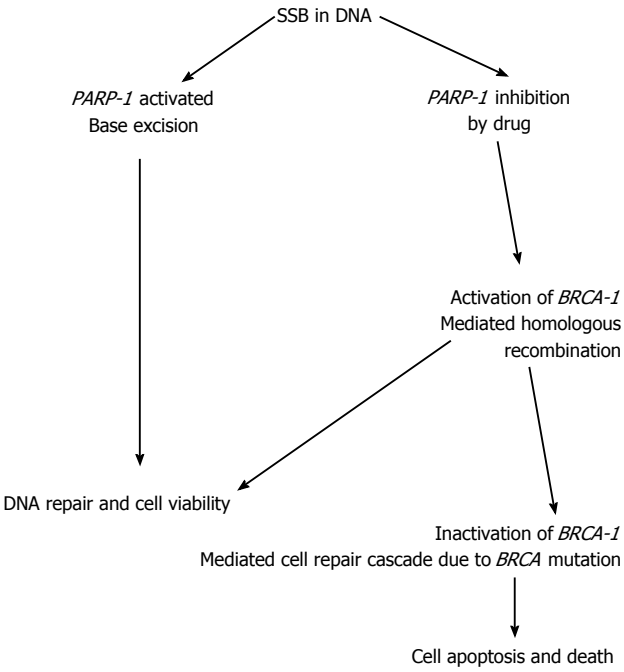

Figure 1 Mechanism of action of poly(ADP-ribose) polymerase-1 inhibitors in triple negative breast cancer. PARP-1: Poly(ADP-ribose) polymerase-1; SSB: Single strand breaks.

Cox-2: Cox is a conversion enzyme of arachidonic acid and prostaglandin. It is a 74kDa protein located in the cell endothelium, reticulum and nuclear membrane. It is expressed by stimuli such as inflammatory response and tumor promoters. In a study by Liu *et al*[114] they observed that 85% of transgenic mice with over expression of Cox developed breast cancer, suggesting the involvement of this enzyme in breast carcinogenesis. Other studies have correlated its expression with invasiveness and metastatic stimuli in breast cancer[115,116]. Approximately 40% of patient with breast cancer over expresses Cox-2. Cox-2 can also be used as a biomarker to assess response to neoadjuvant chemotherapy in breast cancer.

Lymph node status is major of prognostic significance in breast cancer patients. Studies have shown that Cox-2 expression is associated with positive lymph node involvement. So Cox-2 may have some role in lymphangiogenesis. Cox-2 expression has been also correlated to hormone receptors in breast cancer, negative hormone receptors with Cox-2 expression indicate worse prognosis. Cox-2 is correlated to HER2 through Ras/MAPK pathway and it is associated with HER2 over expression[117]. Cox-2 expression is also related to MDR-1, a multidrug resistance gene. Patients with expression of both these are least responsive to chemotherapy. So Cox-2 can be a good biomarker in breast cancer patients with its correlation with size of the tumour, number of nodes involved, hormone receptors and HER2 status[118].

TK: TKs are regulatory proteins that help in the cell growth and differentiation. These proto-oncogenes play an important role in progression and metastasis of cancer cells. They also increase sensitivity of cancer cells once the tumour has been exposed to radiation and chemotherapy through apoptosis[36]. Hence, TKs are of major interest and are subject of many active studies to look targets for therapeutic intervention in many solid tumours. HER2/neu and EGFR are also TKs receptors as discussed above. HER2/neu over-expression is seen in 20%-25% of invasive breast cancers and it is considered a poor prognostic factor. Other TKs over-expressed in carcinoma of the breast are BRK, c-Src, and EGFR[119]. Lack of expression of some of TKs such as Syk and C-kit are also linked to carcinogenesis of breast cancer. TK over-expression in women with breast cancer is have high risk of metastasis. There are many agents that target the phosphorylation of the receptor by acting at TK[120]. TK inhibitors such as imatinib, erlotinib, gefitinib and lapatinib are used for treatment of many solid tumours. Dasatinib and lapatinib are used in treatment of women with HER2/neu positive breast cancer.

Mammalian target of rapamycin: One of the pathway is commonly dysregulated in breast cancer is phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR). Over expression of the PI3K/mTOR is associated with poor response to treatment with hormones and trastuzumab[121]. To overcome endocrine resistance agents such as rapalogs, that efficiently block mTOR-raptor complex 1, can be used along with hormones. However, it has demonstrated variable results in hormone receptor positive metastatic breast cancer[122].

Many targets such as $\alpha V\beta 6$, cyclin E, C-kit, E-cadherin, O^6MGMT, FOXp3, β-blockers, insulin like growth factors, glycoprotein NMB and mitogen-activated protein kinase pathway needs further exploration to dissect TNBC and may possibly identify new biomarkers and targets for therapy.

CONCLUSION

TNBC is the most poorly understood and is refractory to current targeted therapies. It is a cause of significant breast cancer mortality because of very few treatment options. Biomarker may be useful as prognostic or predictive indicators as well as suggest possible targets for novel therapies. Targeted therapy directed against many biomarkers has not shown significant improvement in outcome in TNBC, therefore it is challenging for the clinicians to deal with this distinct disease. The emphasis should be put on research for effective drugs and targets for the treatment TNBC. So, to translate the present knowledge about TNBC into oncological practice, biomarkers/molecules/GEP assays that can truly classify TNBC and can be easily translated to the clinics are necessary.

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REVIEW

Dynamic role of myofibroblasts in oral lesions

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Abstract

Fibroblasts are the most abundant cellular components of connective tissue. They possess phenotypical heterogenicity and may be present in the form of smooth muscle cells or myofibroblasts (MFs). MFs are spindle-shaped cells with stress fibres and well-developed fibronexus, and they display α-smooth muscle actin immunohistochemically and smooth-muscle myofilaments ultrastructurally. MFs play a crucial role in physiological and pathological processes. Derived from various sources, they play pivotal roles not only by synthesizing and producing extracellular matrix components, such as other connective tissue cells, but also are involved in force production. In the tissue remodelling phase of wound closure, integrin-mediated interactions between MFs and type I collagen result in scar tissue formation. The tumour stroma in oral cancer actively recruits various cell types into the tumour mass, where they act as different sources of MFs. This article reviews the importance of MFs and its role in pathological processes such as wound healing, odontogenic cysts and tumours, salivary gland tumours, oral preneoplasia, and oral squamous cell carcinoma. Research oriented on blocking the transdifferentiation of fibroblasts into MFs can facilitate the development of noninvasive therapeutic strategies for the treatment of fibrosis and/or cancer.

Key words: Myofibroblasts; Neoplasm; Fibroblasts; Precancerous lesions; Carcinoma-associated fibroblasts; Precancerous conditions

Core tip: Myofibroblast (MFs) are spindle-shaped cells consisting α-smooth muscle actin myofilament. They have a multicellular origin. MFs of the oral cavity have more contractile ability than dermal fibroblasts in physiologic wound healing. Recently, carcinoma-associated fibroblasts (CAFs) have received considerable attention because of their role in carcinogenesis. Mainly,

transforming growth factor-β released from oral cancer cells is responsible for transforming fibroblasts into CAFs, which leads to tumour progression. However, the role of MFs in oral leukoplakia and oral submucous fibrosis is not completely understood. Understanding the implications of therapeutic approaches for the transdifferentiation of fibroblasts into MFs at different stages of carcinogenesis will facilitate in developing a treatment plan.

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INTRODUCTION

Oral mucosa comprises stratified squamous epithelium, and its underlying connective tissue harbours various mesenchymal cells such as fibroblasts, endothelial cells, and pericytes[1,2]. These cells and their extracellular matrix (ECM) play pivotal roles in cell differentiation and proliferation during tissue development, wound healing processes, and pathological alterations[2,3].

Fibroblasts, the most abundant cellular components of connective tissue, are phenotypically heterogenous and are present in the form of smooth muscle cells or myofibroblasts (MFs)[4]. Gibbiani was the first to observe fibroblasts under an electron microscope and coined the term "myofibroblast"[5].

MFs may be defined as large spindle-shaped cells with stress fibres and well-developed fibronexus. They display α-smooth muscle actin (α-SMA) immunohistochemically and smooth muscle myofilaments ultrastructurally[6,7]. Therefore, MFs, which are found in normal skin tissue; pulmonary septa; and periodontal ligament[8], are unique[4] and owing to their location, they are called as "juxtaparenchymal cells"[4].

MFs show functional heterogenecity through various mechanisms. During mesenchymal-epithelial interactions, they play a pivotal role in organogenesis and morphogenesis. They secrete the components of the ECM and basement membrane[4]. Their myomechanical function is crucial during wound healing[9]. Their role in the carcinogenic process is well-recognised[10]. This article describes the cascade of events pertaining to the role of MFs in wound healing, oral squamous cell carcinoma (OSCC), odontogenic cysts and tumours, and salivary gland tumours.

DISCUSSION

Genesis and identification of MFs

MFs are multicellular in origin (Figure 1). Local fibroblasts, pericytes, endothelial cells, circulating hematopoietic precursor cells, and fibrocytes are various sources of MFs. They are also derived from bone marrow and are known as bone marrow-derived MFs[11]. The main factor responsible for the transition (activation) of fibroblasts to MFs is transforming growth factor-β1 (TGF-β1)[12]. A previous study demonstrated that connective tissue factors (thrombin and endothelin) and platelet-derived growth factor (PDGF) are also responsible for the differentiation of fibroblasts into MFs[11]. According to Werner *et al*[13], keratinocytes play an important role by releasing interleukin-1, activin, and TGF-β1 during the differentiation of fibroblasts into promyofibroblasts into MFs. Thus, three local events are required to generate α-SMA-positive differentiated MFs: (1) the accumulation of biologically active TGF-β1, which enhances the assembly of stress fibres and the formation of fibronexus adhesion complexes; (2) the accumulation of an ED-A splice variant of fibronectin, which are specialised ECM proteins; and (3) the mechanical properties of the ECM and cell remodelling activities, which are responsible for high extracellular stress[9-11]. Carthy *et al*[14] demonstrated that Wnt3a promotes MF-like phenotype formation in cultured fibroblasts. Based on *MF* gene expression patterns, various studies have revealed that distinct subtypes of fibroblasts exist at different sites of the body[14,15]. Nevertheless, an inactive JunD, which protects the cell against oxidative stress, promotes MF differentiation[16]. Their presence at a site may either be pre-existing, or they may originate denovo from the surrounding subpopulation[15].

MFs possess several distinguishing morphologic features[17] and are characterised by the highly contractile α-SMA apparatus[9], which is the most significant marker of myofibroblastic cells. They may express smooth muscle myosin heavy chains and desmin[18]. They also express caldesmon, SM22, and tropomyosin. Under the electron microscope, MFs are large cells with abundant rough endoplasmic reticulum and fibronexuses[5], prominent cytoplasmic actin microfilaments (stress fibres), nonmuscle myosin, and vimentin[17], which are connected to each other by adherens and gap junctions[4,9].

Recently, the 4Ig isoform of the protein palladin in stress fibres was proposed as a new marker of MF differentiation[19]. Conversely, another study suggested that interferon-γ reduces α-SMA expression in smooth muscle cells[18].

Role of MFs in wound contraction

The breach of the epithelial layer is followed by changes that occur in the underlying connective tissue resulting in the loss of tissue homeostasis[1]. Normal wound healing is a well-known phenomenon. It involves a sequence of events including inflammation, proliferation, and tissue remodelling[18,20,21]. Wound closure involves connective tissue deposition, epithelization, and contraction[22] (Figure 2).

Wound contraction is mainly carried out by MF, a specialised contractile fibroblast[22]. Initially, small tractional forces exerted by the ECM facilitate the formation of protofibroblasts, which are composed

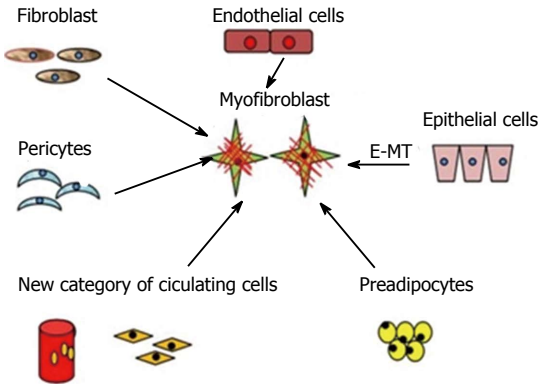

Figure 1 Origin of myofibroblasts-multicellular origin. Multicellular origin of myofibroblasts: Fibroblast, pericytes, endothelial cells, circulating hematopoietic precursor cells and fibrocytes can transform into myofibroblasts. E-MT: Epithelial-mesenchymal transition.

of cytoplasmic actin components and devoid of the contractile apparatus and α-SMA. Protofibroblasts migrate to the wound site by acquiring a migratory phenotype at the fibronectin-fibrin wound interface. At the site, protofibroblasts generate comparably small traction forces. Cytokines, such as PDGF, granulocyte-macrophage colony-stimulating factor (GM-CSF), heparin, integrin[20], and TGF-β, and existing tractional forces stimulate protofibroblast differentiation through α-SMA expression, leading to their transformation into MFs. A study conducted on the role of tenacin in wound contraction demonstrated that increased tenacin expression correlated with MF differentiation[23]. However, interferon-γ, a basic fibroblast growth factor (bFGF), prostaglandin E2, and high cell density inhibit the differentiation of protofibroblasts to MFs[20]. After activation, MFs initiate the synthesis of a new collagen-containing matrix that consists proteoglycans and glycosaminoglycans (highly hydrated molecules)[1,22,24]. They produce tractional forces at the margins for wound contraction, which is known as tractional remodelling[22].

MFs generate forces in two ways. Initially, actin filaments present within the cell form a fibronexus by connecting intracellular actin and extracellular fibronectin fibrils using integrins. Integrins mediate the reorganisation and contraction of collagen matrices with the help of fibroblasts[20]. A study on α1 integrin knockout mice revealed impaired wound healing[20]. The assembly formed by MF with integrin and the actin filament is responsible for the "mechano-transduction system", which produces a high degree of tractional forces[9]. Later, MFs connect to each other through gap junctions to form a "multicellular contractile unit". They again exert a force on the ECM by implicating the use of this unit[9,22]. Both mechanisms exert a high level of tractional forces for wound closure.

After complete wound closure and re-epithelization, the number of MFs decreases either by reverting to the quiescent form or by undergoing apoptosis[1,9,25]. In the tissue remodelling phase, integrin-mediated interactions between MFs and collagen I results in scar tissue formation[20].

Wound healing in the oral cavity essentially occurs without scarring and is faster than skin healing[20]. Fibroblasts in the oral mesenchyme possess a unique phenotypical character by constitutively expressing elevated α-SMA levels, along with a higher capacity to contract collagen gel and a higher replicative potential than dermal fibroblasts[17], ultimately leading to a "scar-free" healing process. Factors, such as epidermal growth factor, vascular endothelial growth factor, bFGF, and insulin-like growth factor, present in saliva and crevicular fluid are responsible for wound healing in the oral cavity[20].

MFs in oral leukoplakia

Leukoplakia is the most common potentially malignant disorder of the oral mucosa[26]. Studies have been conducted on various histopathological grades of oral leukoplakia (OL), but failed to establish a conclusive relationship between OL and MFs. MFs were not found in the stroma under dysplastic epithelium[16]. Myofibroblastic differentiation depends on the following factors: (1) Neoplastic microenvironment, which releases various growth factors[27]; (2) Genetically altered epithelium (carcinomatous epithelium), which is responsible for the inductive effect on the underlying stroma[28]; and (3) Epithelial-mesenchymal interaction (EMI), which plays a role in "epithelial invasion"[16,19].

However, these factors were absent in various grades of epithelial dysplasia[16,19,27,29]. A study conducted by de-Assis *et al*[29], demonstrated that OL was not associated with MFs because MFs were not found in any of the samples. Therefore, it was hypothesised that myofibroblastic differentiation was entirely dependant on oral carcinoma development and, simultaneously, on the contact of cancer cells with stromal cells achieved by invading the epithelial cells[16].

MFs in oral submucous fibrosis

The most chronic and functionally hampering condition of oral cavity is oral submucous fibrosis (OSMF). OSMF is an abnormal healing process in response to the chronic mechanical and chemical irritation caused by chewing areca nuts[30]. The cellular mediator responsible for fibrosis is MF, which serves as collagen-producing cells when activated[31]. Continuous MF presence stimulates an abnormal repair mechanism, leading to excessive contraction and ECM secretion, subsequently causing fibrosis[30]. It was proposed that in fibrosis, MFs acquire an immune-privileged cell phenotype, which helps them to evade apoptosis and allows their uninterrupted accumulation[31]. A study suggested that OSMF could represent failed wound healing after chronic and sustained injury. Studies have proposed that fibrosis in OSMF could result from a hypersensitivity response caused by arecoline and a juxta-epithelial inflammatory response, which initiates a defective inflammatory response, activates fibroblasts, and culminates in fibrosis[30,32]. In addition, MFs could be used as potential markers for evaluating disease

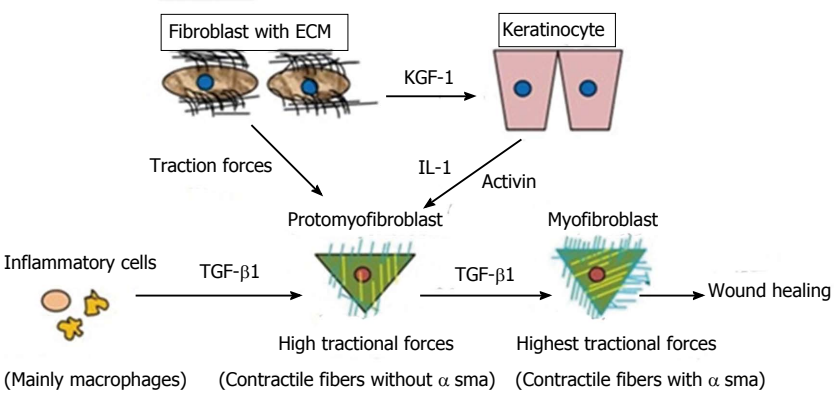

Figure 2 Differentiation of promyofibroblast to myofibroblast. By getting stimulus from various cytokines profibroblasts (promyofibroblasts) transforms into myofibroblasts by expressingα-smooth muscle actin. TGF-β1: Transforming growth factor-β1; ECM: Extracellular matrix; IL-1: Interleukin-1; KGF-1: Keratinocyte growth factor-1.

severity because a progressive increase in MFs from the early to the advanced stages was observed[30].

The malignant transformation rate of OSMF ranges from 7% to 13%[33]. Dyavanagoudar[33] hypothesised that precancerous epithelial cells of OSMF acquire multiple genetic mutations in mesenchymal-epithelial crosstalk, and the associated stroma becomes activated and expresses SMA markers. These cells express ECM proteins and growth factors. These factors enhance and support tumour cell survival in an autocrine and paracrine manner, respectively. In contrary, Moutasim *et al*[34] demonstrated that αvß6 was markedly upregulated in OSMF by oral keratinocytes in an "*in vitro*" study. The results of the study also revealed that arecoline (the major alkaloid of areca nut) upregulated keratinocyte αvß6 expression, which induced oral fibroblasts to transdifferentiate into MFs and resulted in the upregulation of genes associated with tissue fibrosis (Figure 3). Blocking these specific integrins can develop novel therapies for such fibrotic conditions[35].

MFs in OSCC

Stromal changes in wound healing and tumourogenesis depend on epithelial homeostasis[1]. Changes in epithelial homeostasis lead to changes in the underlying connective tissue stroma. This stroma is called reactive or desmoplastic stroma. Reactive stroma plays a significant role in the growth and progression of carcinoma because malignant epithelial cells require support from the surrounding stroma to promote tumorigenic progression[3]. Carcinoma-associated fibroblasts (CAFs) or tumour-associated fibroblasts or MFs are found in considerable quantities in such stroma[36] (Figure 4).

CAF origin is controversial. A study demonstrated that CAF originate from cancer stem cells, which are a small subpopulation of the tumour stroma[37]. Another study demonstrated that 60% CAF originate from bone marrow-derived mesenchymal cells[36]. Some additional sources may be resident fibroblasts, endothelial cells, pericytes, smooth muscle cells, and preadipocytes. Studies have demonstrated that the mutual paracrine effects between oral cancer cells and normal fibroblasts are responsible for the transdifferentiation of normal fibroblasts into malignant fibroblasts. CAF genesis is also related to an EMI in the stromal population or a possible transdifferentiation of the malignant epithelial cells into MFs during oral carcinogenesis[1,19]. The tumour stroma continues to remodel itself during tumour progression and actively recruits various cell types into the tumour mass where they act as different sources of MFs[3]. TGF-β is the main factor responsible for fibroblastic differentiation leading to activated tumour MFs. It is a main mediator found in the saliva and is expressed by cancer cells. A study demonstrated that the tension exerted in the tumour stroma is also responsible for the transformation of fibroblasts into MFs[1]. Keratinocytes also seem to be responsible for forming tumour-associated fibroblasts[33].

Morphologically tumour fibroblasts differ from normal MFs in their abundant rough endoplasmic reticulum, Golgi apparatus, fibronectin fibrils, and fibronexuses on the cell surface[5]. They also differ in their contractile property, MFs because they can exert more contractile force which is responsible for stiffness in the advanced stages of the neoplasm[1]. MFs secrete several enzymes such as stromelysins and matrix metalloproteinases (MMPs- 1, 2, 3, 9, 13, and 14)[1], which cause ECM degradation. They also release different growth factors such as PDGF, bFGF, keratinocyte growth factor, stem cell factor, epidermal growth factor, GM-CSF, and other cytokines[25]. They also secrete matricellular proteins including CCN2; collagens; tenascins C and FN; and elastins[1]. MFs promote tumour cell migration on tenacin[38]. Hence, MFs play an important role in tumour progression by invading the tumour stroma and consequently remodelling the ECM by forming more desmoplastic responses[25,38]. Most importantly, they secrete fibroblast-associated proteins (FAP). The loss of FAP is associated with inhibition of tumour cell progression and decrease in MF quantity and blood vessel density in the tumour[16].

MFs function as "sentinel cells" by acting as immunoregulatory cells in the stroma, by reducing the physical contact between cancer and immune cells, which is

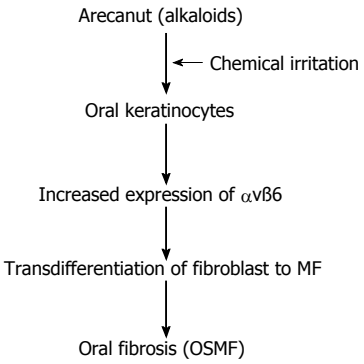

Figure 3 Transdifferentiation of fibroblasts into myofibroblasts. Major alkaloid of areca nuts, up-regulates keratinocyte αvß6 expression which induced transdifferentiation of oral fibroblasts into MF. OSMF: Oral submucous fibrosis.

imperative for cancer cell destruction[25].

Several MFs in the tumour stroma are often associated with high-grade malignancies. They promote tumour progression through "neo-angiogenesis" by releasing growth factors, such as fibroblast growth factor-2[3]. A study demonstrated that the most migratory and invasive behaviour of tumour cells correlated with the presence of MFs at the invasive tumour front in OSCC because this precedes the invasive stage of the cancer[39,40]. In addition, abundant MFs in the tumour stroma correlates with a higher tumour incidence, specifically in patients aged below 60 years[19].

MFs are present in the OSCC stroma in two dominant patterns, spindle pattern (MFs are arranged in rows with a few cells around the neoplastic islands) and network pattern (several abundant layers of MFs around the neoplastic islands). The network pattern fibroblasts are exceptionally abundant and occupy almost the entire tumour stroma, whereas the spindle pattern includes spindle cells that are located at the periphery in 1-3 concentric layers[16]. Studies conducted by Seifi *et al*[39] and Kawashiri *et al*[41] have demonstrated that the presence of MFs and the arrangement of MFs in the tumour stroma play a role in invasion. They observed that MFs arranged in the network pattern caused tumour invasion and not those arranged in the spindle pattern. They also noted that tumour desmoplasia is associated with aggressive cancer.

Functional similarities and differences between wound healing and tumorigenesis

Morphological similarities exist between the tumour stroma and granulation tissue following wound healing. A wound is disruption of an anatomical structure, such as an epithelial membrane, and healing is the restoration of structure and function[41] (Figure 5).

Wound healing and tumour progression are loosely related in terms of MFs[1]. In wound healing, changes in the underlying stroma occur with a breach in the normal lining epithelium. The appearance of a break causes changes in the underlying connective tissue stroma. Inflammatory cells release TGF-β1, which stimulate the differentiation of fibroblast into proto-myofibroblast and finally into MFs[9]. MFs later release MMPs and growth factors, which lead to matrix degradation and new blood vessel formation (angiogenesis), respectively. MFs also cause wound contraction and undergo apoptosis. However, in the last stage of wound healing, a defect in the apoptosis of MF and the persistence of MF results in an hypertrophic scar tissue[42].

A tumorigenic process involves acquiring multiple genetic mutations. In the normal cell ecosystem, a continuous cross-talk between epithelial cells and stroma occurs. Epithelial cells acquiring neoplastic properties result in altered stromal compartment. Neoplastic cells release TGF-β1 and PDGF, which result in the emergence of CAF[3,10,43]. The petite concentration of TGF-β1 increases (femtomolar to picomolar) as the fibroblasts approach the cancer cells and transdifferentiate into MFs[43]. CAF release growth factors and cause angiogenesis similar to wound healing[10]. However, in carcinogenesis, MMPs (1, 2, 9 and 13) liberated by CAF are approximately double than the normal fibroblasts[44]. These factors together promote tumour progression and invasion[43].

Thus, the loss of epithelial homeostasis is a common trigger for the stromal reaction against tumours and for normal wound healing[1]. In both processes, growth factors and MMPs liberated by MFs are responsible for progression.

In wound healing, MFs transiently acquire the phenotype[1], which persists during fibrosis in the tumour environment because of an imbalance between the apoptosis and the proliferation of transformed cells leading to disease progression[40].

MFs in odontogenic cyst and tumour

Smith was the first to question the relationship between MFs and the aggressive behaviour of a neoplasm[45]. Rothouse reviewed the ultrastructural features of ameloblastoma and found that the stromal component of an ameloblastoma is composed of MFs along with associated collagen and basal lamina material[46]. Vered *et al*[43] studied the number of MFs in a solid ameloblastoma and parakeratinised odontogenic keratocyst to find that the mean number of MFs in the odontogenic lesions was the same as that in OSCC[43]. The number of MFs was high in these lesions, which were responsible for aggressive and invasive behaviour. They explained that the epithelium of parakeratinised odontogenic keratocyst and solid ameloblastoma behave like the OSCC epithelium by releasing more TGF-β1 and simultaneously modulating stromal MFs, making the tumour more aggressive and invasive[43]. This explains the biologic behaviour of these odontogenic lesions. Another study demonstrated the invasive behaviour of a recurrent infiltrative ameloblastoma because of a high number of MFs in stroma[46]. Thus, the presence and the frequency of MFs in the stroma possibly determine the biologic behaviour of the lesions.

Odontogenic myxoma, which is considered to be a slow-growing invasive tumour, showed abundant MFs in

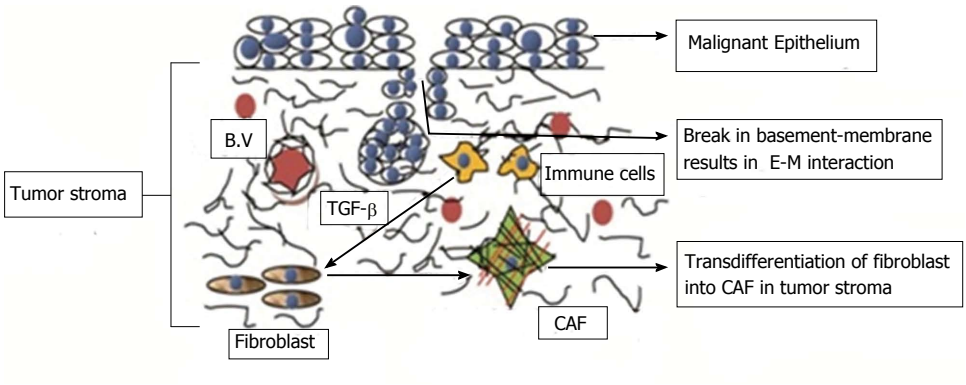

Figure 4 Transdifferentiation of fibroblast into carcinoma associated fibroblast in oral squamous cell carcinoma. Mutual paracrine effect between oral cancer cells and normal fibroblasts is responsible for transdifferentiation of the latter into malignant fibroblasts. CAF: Carcinoma associated fibroblast; TGF-β: Transforming growth factor-β; B.V: Blood Vessels; E-M: Epithelial-mesenchymal.

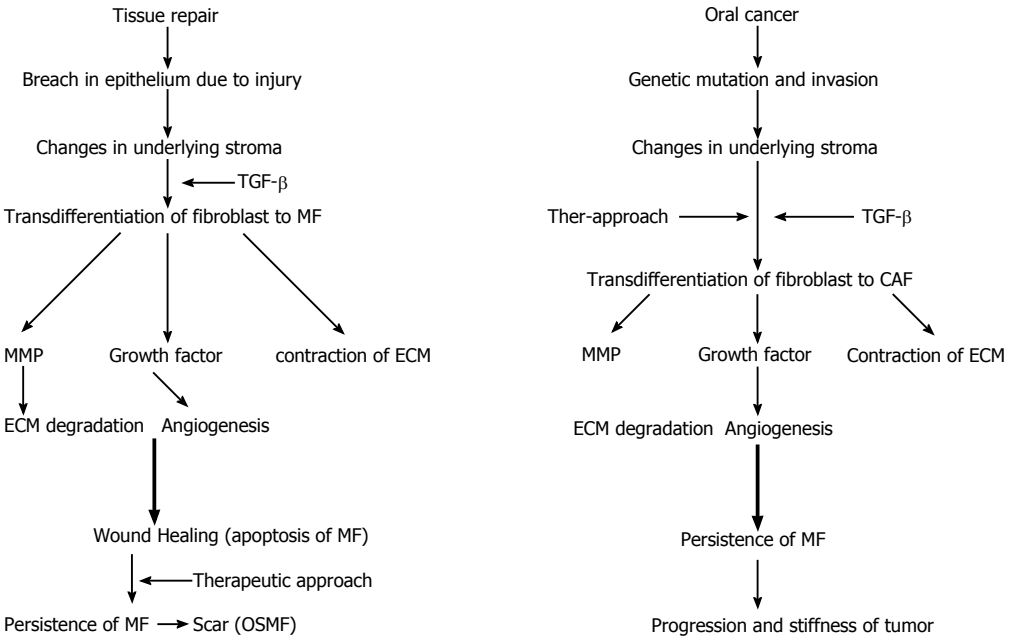

Figure 5 Functional similarities and differences between wound healing and tumerogenesis. Wound healing and tumour progression phenomenon in relation with MF. TGF-β: Transforming growth factor-β; ECM: Extracellular matrix; CAF: Carcinoma-associated fibroblasts; OSMF: Oral submucous fibrosis; MMP: Matrix metalloproteinase; MF: Myofibroblast.

its stroma associated with its invasive behaviour. Effiom *et al*[47] hypothesised that MFs present in the stroma modify the ECM by releasing various cytokines, which are responsible for epithelial invasion.

Various authors have studied the relationship between different cysts and MFs in their stroma. The increased MFs in the stroma directly related to a more aggressive behaviour of the odontogenic cysts[48], radicular cysts[49], dentigerous cysts[49], and keratocystic odontogenic tumours[49]. The results indicated that MFs were present in a decreasing order in the cyst stroma of keratocystic odontogenic tumours, dentigerous cysts, and radicular cysts. However, the results were not statistically significant. Some authors suggest that the presence of MFs in the cyst wall might be part of a homeostatic response to the distension caused by cyst enlargement[50].

MFs in salivary glands

The tumorigenic role of MFs in the salivary gland is controversial. Studies conducted on MFs in the tumour stroma of several salivary gland tumours, such as carcinoma ex pleomorphic adenoma, mucoepidermoid carcinoma (MEC), and adenocarcinoma, revealed the presence of tumour MFs at the tumour front in all the malignant lesions, which further correlated with the invasiveness of the cancer cells[40]. Gupta *et al*[51] demonstrated that a high MF density in adenoid cystic carcinoma and MEC at tumour front, contributing to the aggressiveness of the lesions, whereas a moderate MF density was observed in polymorphous low grade adenocarcinoma. Conversely, Soma *et al*[52] demonstrated that MFs inhibit tumour growth because they found more MF presence at the tumour border in benign lesions (pleomorphic adenoma) than that

in malignant lesions. Thus, they postulated that the MFs present at the periphery of benign lesions were responsible for containing the tumour, whereas the absence of MFs in malignant tumours was responsible for the progression of the tumour cells owing to no containment of the tumour[52]. Sobral *et al*[53] reported similar findings. They demonstrated that MF was higher in low grade MEC than the intermediate and high grade suggesting that the inflammatory infiltrate in the tumour stroma causes the cessation of MF differentiation. With the increasing grades of MEC, the inflammatory infiltration increased, and therefore, the number of MF decreased, leading to a poor prognosis[53,54].

The role of MFs in the pathogenesis of mucocele or chronic sialadenitis was not found. It was postulated that MFs may play a "supportive muscular role" around the cystic wall of the mucous retaining cyst and distended excretory duct[55].

CONCLUSION

MFs are known to contribute to the biological behaviour of various lesions. They actively participate in diseases characterised by tissue fibrosis because of their ability to secrete and degrade ECM components. MFs also play a role in ECM remodelling induced by tumour cells. It, thus, creates a permissive or suitable environment for tumour progression. Therefore, MFs are unique contractile cells that play a role in not only growth, development, and wound healing but also in inflammation, fibrosis, and tumour progression. Benefitting from their advantages in physiologic processes and blocking the processes leading to the causation and progression of a disease are the need of the hour. Additional knowledge and clinical studies involving this unique cell may provide us with an effective target for cancer therapy. Limited studies on oral lesions calls for further research for understanding the molecular mechanisms of MFs in the progression of these lesions.

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Pelvic radiation disease: Updates on treatment options

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Abstract

Pelvic cancers are among the most frequently diagnosed neoplasms and radiotherapy represents one of the main treatment options. The irradiation field usually encompasses healthy intestinal tissue, especially of distal large bowel, thus inducing gastrointestinal (GI) radiation-induced toxicity. Indeed, up to half of radiation-treated patients say that their quality of life is affected by GI symptoms (*e.g.*, rectal bleeding, diarrhoea). The constellation of GI symptoms - from transient to long-term, from mild to very severe - experienced by patients who underwent radiation treatment for a pelvic tumor have been comprised in the definition of pelvic radiation disease (PRD). A correct and evidence-based therapeutic approach of patients experiencing GI radiation-induced toxicity is mandatory. Therapeutic non-surgical strategies for PRD can be summarized in two broad categories, *i.e.*, medical and endoscopic. Of note, most of the studies have investigated the management of radiation-induced rectal bleeding. Patients with clinically significant bleeding (*i.e.*, causing chronic anemia) should firstly be considered for medical management (*i.e.*, sucralfate enemas, metronidazole and hyperbaric oxygen); in case of failure, endoscopic treatment should be implemented. This latter should be considered the first choice in case of acute, transfusion requiring, bleeding. More well-performed, high quality studies should be performed, especially the role of medical treatments should be better investigated as well as the comparative studies between endoscopic and hyperbaric oxygen treatments.

Key words: Pelvic radiation disease; Radiation-induced proctopathy; Radiotherapy; Gastrointestinal toxicity; Sucralfate; Metronidazole; Probiotics; Argon plasma coagulation; Hyperbaric oxygen; Formalin

Core tip: Radiotherapy is frequently employed as part of the multimodal treatment of pelvic cancers. Despite recent advances in irradiation techniques, acute and late- onset radiation-induced gastrointestinal toxicity, also known as pelvic radiation disease, is still being frequently reported. This review provides an up-to-

date summary on medical and endoscopic approaches that have been evaluated with treating intent, focusing on the best available evidence, primarily randomized controlled studies.

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INTRODUCTION

Pelvic cancers are among the most frequently diagnosed neoplasms[1]. The employment of radiation therapy as part of a multidisciplinary treatment for pelvic malignancy has progressively increased in recent years[2], as it is estimated that over 200000 patients in the United States receive pelvic or abdominal radiation therapy annually. The irradiation field usually encompasses healthy intestinal tissue, especially of distal large bowel, thus inducing gastrointestinal (GI) radiation-induced toxicity. Indeed, up to half of radiation-treated patients say that their quality of life is affected by gastrointestinal symptoms[3]. Recently, the constellation of gastrointestinal symptoms - from transient to long-term, from mild to very severe - experienced by patients who underwent radiation treatment for a pelvic tumor have been comprised in the definition of pelvic radiation disease (PRD)[3]. Radiation toxicity is defined as acute when occurring during radiotherapy or within 3 mo, while it is considered as chronic when developing after longer period of time. Among the most frequently reported symptoms are diarrhea, urgency, rectal bleeding and fecal incontinence[4].

The type of irradiation technique has been recognized as an influential factor for the development of PRD[5]. It is important to notice that even the most recent radiation procedures, such as intensity-modulated radiotherapy, have reduced but not completely annulled the occurrence of GI radiation-related toxicity[6]. Moreover, the prolonged survival of this category of patients will undoubtedly increase the risk of developing PRD over time. Thus, a correct and evidence-based therapeutic approach of patients experiencing gastrointestinal radiation-induced toxicity is mandatory. Therapeutic non-surgical strategies for PRD can be summarized in two broad categories, *i.e.*, medical and endoscopic. Over years, a number of medical treatments have been investigated, such as aminosalicylates, sucralfate, antibiotics, probiotics, steroids and hyperbaric oxygen therapy. Endoscopic treatments have been explored too, including argon plasma coagulation, formaline application, radiofrequency, criotherapy and band ligation.

In the current review, we provide a critical appraisal of the efficacy of the treatment options for radiation-induced gastrointestinal toxicity.

Pathogenesis

The occurrence and severity of radiation-induced gastrointestinal toxicity depends upon several factors. Therapy-related factors include radiation dose, volume of irradiated bowel, time- and dose-fractioning parameters and concomitant employment of chemotherapy. Patient-related factors include smoking, body mass index, previous abdominal surgery and comorbidities like inflammatory bowel disease, diabetes and collagen vascular disease[7-13].

Traditionally, the development of radiation enteropathy was explained through the "target cell" theory, which addressed early pathology to the epithelial injury, while fibroblast and endothelial cell damage was accounted for late-onset harm[14]. In recent years, the above-mentioned theory has been questioned, and other factors have been taken into account. For instance, the enteric nervous system is the second largest nervous system of human body, and it has been pointed out as capable to regulate radiation enteropathy development[15]. It has also been demonstrated that the gut microbiota, consisting of about 100 trillion bacteria, influences radiation-induced damage[16]. Thus, the understanding of PRD pathogenesis has gone far beyond the single "target cell" concept, and considers intestinal toxicity as the result of multiple interactions between epithelial injury, gut microvasculature, enteric nervous system, and gut microbiota[17].

Acute and chronic gastrointestinal toxicity have a different pathogenesis[18]. Indeed, acute PRD is due to an acute inflammatory response, whilst chronic, late-onset disease is mainly mediated by vascular sclerosis and fibrosis[19]. However, acute and chronic radiation toxicities are not independent events, as it is underlined by the consequential late effect theory: indeed, late injury is more likely to develop when severe acute toxicity exists[20,21]. Recent studies have added complexity to these models[17], however a deeper discussion on the pathological basis of PRD is beyond the purpose of this review and we invite to consider for this purpose the review by Hauer-Jensen *et al*[17].

Treatment options

Medical treatment, hyperbaric oxygen therapy and endoscopic approaches represent the mainstay for treating pelvic radiation-induced disease. However, the existing evidence on such approaches for treating PRD cannot be judged as of high quality, due to few and low-quality randomized controlled trials (RCTs), high clinical and methodological heterogeneity, small sample sizes and short periods of follow-up[22].

MEDICAL TREATMENT

Medical therapy should represent the first step in the management of radiation-induced pelvic radiation disease. Over years, a number of medical treatments

Table 1 Medical strategies for treating pelvic radiation disease

Medical treatments	Acute PRD	Chronic PRD	Notes
Topical sucralfate	N	Y	Twice-daily enema with two 1 g sucralfate tablets mixed with 4.5 mL of water is effective for chronic rectal bleeding
Metronidazole	N	Y	3 × 400 mg/d of metronidazole for up to 12 wk is effective for chronic rectal bleeding and diarrhea
Probiotics	Y	N	3 sachets/d of *Lactobacillus rhamnosus* for at least 1 wk is effective for acute diarrhea
Mesalazine	N	N	No recent RCTs available; one prospective study showed that combined oral and topic mesalazine was effective for chronic rectal bleeding
Corticosteroids	N	N	RCTs have not shown a substantial improvement with steroids administration
Hyperbaric oxygen	N	Y	At least 30 sessions (up to 100) are effective for chronic rectal bleeding not responding to medical treatment

Y: Evidence supports treatment; N: Evidence does not support treatment; PRD: Pelvic radiation disease; RCT: Randomized controlled trial.

have been investigated, such as aminosalicylates, sucralfate, antibiotics, probiotics, steroids and hyperbaric oxygen therapy (Table 1).

Radiation-induced injury has been misleadingly referred to as *proctitis,* though inflammation has a non-central role in the pathogenesis of the disease. Thus, anti-inflammatory agents (steroids and 5-aminosalycilic acid) have traditionally been proposed as first-line treatment, with inconsistent results confirmed by a recent systematic review[23]. In fact, only sucralfate and metronidazole have clearly shown to be effective for treating symptoms of PRD, and the role of probiotics is supported by one RCT only.

Sucralfate

Rationale: Sucralfate is an alkaline aluminum hydroxide of sulfated sucrose. The rationale for the administration of sucralfate in the treatment of PRD lies on its supposed property to protect mucosa by forming a viscous superficial coating and to stimulate mucosal healing by its angiogenic action[24,25].

Evidences: According to published prospective studies - including one small, non-placebo controlled randomized controlled trial - topical sucralfate is effective in the treatment of PRD, as it significantly reduces the entity of rectal bleeding[26-29]. Indeed, patients experiencing symptoms improvement ranged from 73% to 100% of considered cohorts, after a follow-up period between four and six weeks. However, when surveillance interval was expanded, symptoms recurred in 10%-20% of patients[27,28]. Oral sucralfate was evaluated by one randomized, placebo-controlled trial and did not show to improve symptoms of PRD when added to endoscopic argon plasma coagulation[30].

Based on the available evidence, topical administration of sucralfate should be considered as one of the first-line treatments of radiation-induced rectal bleeding. The topical administration is the only way of assumption that showed to be effective for PRD[26,27,29]. Sucralfate can be administered twice daily as a retention enema prepared by patients themselves, using two 1 g sucralfate tablets mixed with 4.5 mL of water in an enema applicator and producing a low-volume paste[29].

Metronidazole

Rationale: Metronidazole is a bactericidal agent that kills anaerobic and microaerophilic bacteria, which contribute to hypoxia, and also has an immunomodulator effect; these two actions may reduce the risk of rectal bleeding and help in the management of PRD.

Evidences: According to RCT-based evidence, metronidazole is effective in treating chronic rectal bleeding and diarrhea[31,32]. Cavcić *et al*[31] randomized 60 patients with radiation-induced rectal bleeding, diarrhea and ulcerations to receive metronidazole (3 × 400 mg/d orally), mesalazine (3 × 1 g/d orally) and betamethasone enema (once a day during 4 wk) or only the combination of mesalazine and betamethasone. After 12 mo of follow-up evaluation, a significant reduction in the incidence of rectal bleeding and diarrhea was found in the metronidazole group.

Sahakitrungruang *et al*[32] enrolled in a RCT 50 patients with chronic radiation-induced PRD; patients were randomized to daily colonic irrigation plus metronidazole (3 × 500 mg/d orally) and ciprofloxacin (2 × 500 mg/d orally) for a week, or to receive 4% formalin by using proctoscopy. Outcomes were evaluated after 8 wk, showing a significant improvement in rectal bleeding, urgency, diarrhea in patients treated with metronidazole.

At the present day, these two studies represent the only RCTs showing the efficacy of metronidazole in the management of pelvic radiation disease. Other studies are recommended in order to confirm the results already achieved.

Based on the existing RCT-based evidence, metronidazole can be administered orally (3 × 400 mg/d) from 1 wk up to 12 wk. Metronidazole can be considered as a safe drug. Skin rash, nausea and vomiting are the most frequently reported side effects[32].

Probiotics

Rationale: Probiotics are defined as living microorganisms that confer a health benefit to the host when administered in adequate amounts[33]. They mainly include lactobacilli and bifidobacteria strains. The possible mechanism of action has been investigated

in various studies. Probiotics seems to have a strong immunomodulation effect by acting on epithelial cells, dendritic cells, monocytes/macrophages and lymphocytes. They also have antimicrobial activity against pathogenic bacterial strains, which is mediated by the reduction of pH, secretion of antimicrobial peptides, inhibition of bacterial invasion and adhesion to the gut epithelium[34]. They enhance barrier integrity and function, also by improving the production of short chain fatty acids, in particular butyrate[35].

In conclusion, probiotics have the potential to maintain or restore the gut microflora during and after radiation therapy, especially reducing the incidence of radiation-induced diarrhea[36].

Evidences: Up to now only one RCT has been performed and showed that probiotics are effective in treating acute diarrhea[37]. More in details, Urbancsek *et al*[37] performed a randomized, placebo-controlled, double-blind trial recruiting 205 patients with diarrhea lasting for at least 2 wk and developed within 4 wk from radiotherapy for pelvic cancers. The efficacy was inferred through the need of rescue medication per patient. After a 1 wk period of treatment, the active group required antidiarrheal drugs less frequently than placebo group, although the difference was not statistically significant. Number of bowel movements, diarrhea grading and stool consistency were also evaluated as secondary end-points, and the active group showed a significant improvement in patients' diarrhea rating and in stool consistency.

According to Urbancsek *et al*[37] *Lactobacillus rhamnosus* can be administered orally as 1.5 g sachets, three time a day, for at least one week. Probiotics, regarded as drugs or only food supplementation, can be considered as safe. No serious adverse drug reactions were reported[37].

Aminosalicylates

Rationale: Aminosalicylates are compounds that contain 5-aminosalicylic acid (5ASA), which is a potent inhibitor of the synthesis and release of proinflammatory mediators (*e.g.*, nitric oxide, leukotrienes, thromboxanes, and platelet activating factor) and also inhibits the function of several cells implicated in the acute inflammatory and immune response (*e.g.*, natural killer cells, mast cells, neutrophils, mucosal lymphocytes, and macrophages)[38]. Aminosalicylates are currently available as pro-drugs (sulfasalazine) and active compound (mesalazine). As eicosanoid inflammatory mediators are the main mediators in the pathophysiology of acute, early-onset PRD[39], the administration of aminosalicylates might be effective in reducing inflammation and therefore improve radiation-induced symptoms.

Evidences: Current evidence on the role of mesalazine in the treatment of PRD is scanty. Indeed, only one randomized, controlled trial has been performed so far, showing that mesalazine significantly improved symptoms such as diarrhea, abdominal pain and flatulence[40]. However, radiotherapy techniques have completely changed since the 70 s, thus the results of the above mentioned trial might not be suitable to present day. One prospective study assessed the efficacy of combined oral and topical mesalazine in 23 patients with chronic PRD, and found that mesalazine significantly improved rectal bleeding, but not other radiation-related symptoms (*i.e.*, pain, tenesmus and stool frequency) after 4 wk of treatment[41].

Current evidence does not support mesalazine routine use for the treatment of acute nor chronic PRD. However, a 4 wk treatment of mesalazine, once daily as a 1 g rectal suspension, might be considered in patients referred for chronic rectal bleeding developed after radiation treatment, as second-line therapy[41].

Corticosteroids

Rationale: Corticosteroids have many metabolic and physiological effects. In fact, they wield anti-inflammatory action by inhibiting the arachidonic acid cascade, blocking cytochine release and production, inhibiting histamine release and activation of macrophages and finally by stabilizing cell membranes[42]. Since the first phase of PRD development is an inflammatory based process, all the effects of corticosteroids might play a role in the early phases.

Evidences: As far as RCT-based evidence is considered, corticosteroids have not clearly shown to induce substantial benefits for treating pelvic radiation disease[31,42,43]. Cavcić *et al*[31] found that the addiction of oral metronidazole to mesalazine and betamethasone enema significantly improved rectal bleeding and diarrhea, therefore suggesting that metronidazole may have synergistic effects with steroids.

Kochhar *et al*[26] performed a double-blind controlled trial comparing sulfasalazine (500 mg three times a day) plus prednisolone (20 mg) enemas *vs* sucralfate enemas (2 g twice a day) plus oral placebo. Thirty-seven patients were enrolled and the treatment was continued for 4 wk. After the follow-up period, the sucralfate group showed a significantly better response as assessed clinically (94% *vs* 53%), thus the authors concluded that both treatment regimens were effective in the management of radiation proctopathy, though sucralfate enemas were better tolerated and had a better clinical response[26]. However, this study had a small sample size, with a follow-up period of 4 wk only, therefore detracting from any relevant conclusion.

Rougier *et al*[42] compared two different corticosteroid enemas, randomizing patients to receive either betamethasone enema (5 mg twice a day) or hydrocortisone acetate foam (90 mg twice a day). At the end of the treatment period, there was a non-significant reduction of rectal bleeding (38% *vs* 21%) in

Table 2 Endoscopic approaches for treating pelvic radiation disease

Endoscopic approaches	Rectal bleeding	Notes
Argon plasma coagulation	Y	Treatment of choice when clinically significant rectal bleeding occurs
Formalin	Y	Alternative to APC, but more prone to complications and requires more skilled endoscopist
Radio frequency ablation	N	No RCT available; possibly effective but more expensive than other treatments
Cryoablation	N	No RCT available; risk of cecal perforation
Rectal band ligation	N	Anecdotic case report

Y: Evidence supports treatment; N: Evidence does not support treatment; RCT: Randomized controlled trial; APC: Argon plasma coagulation.

favour of hydrocortisone, and betamethasone enemas were poorly tolerated in 10 of 14 patients compared with 2 of 16 patients in the hydrocortisone group. However, no firm conclusion can be drawn from this study, as patients in the betamethasone group suffered from a more severe disease and the follow-up period was too short.

Hyperbaric oxygen

Rationale: As the pathogenesis of chronic, late-onset pelvic radiation disease is mainly mediated by mucosal ischemia due to vascular sclerosis and fibrosis, and by oxidative stress, hyperbaric oxygen (HBO) therapy has been proposed. Indeed, HBO acts by inducing regrowth of injured vascular endothelial cells and epithelial cells, both directly and through stimulation of connective tissue elements[43]. HBO also improves the activity of radioprotective antioxidant enzymes and reduces free-radical damage[44,45].

Evidences: A systematic review of several case-series concluded that HBO therapy improved symptoms of radiation-induced GI toxicity in nearly 60% of patients and induced symptoms remission in 35% of patients[46]. So far, only one randomized controlled trial has been performed, comparing HBO therapy at 2.0 absolute atmospheres to normal air at 1.1 absolute atmospheres in 120 patients with chronic rectal bleeding refractory to medical treatment[47]. In this study, Clarke *et al*[47] found that HBO therapy significantly improved late-onset rectal bleeding, yielding a 32% absolute risk reduction and a number needed to treat equal to 3. However, the crossover design of the trial did not allow concluding whether symptom improvement was maintained long-term in the HBO therapy arm.

HBO should be regarded as the treatment of choice in case of chronic, radiation-induced rectal bleeding not responding to medical treatment or as second-line option in case of endoscopic failure. HBO can be considered as a relatively safe therapy, as its reported side effects were mild, transitory and self-limiting. The most frequently reported side effects are otic barotrauma, confinement anxiety and temporary myopia[47,48]. Of note, none of these side effects led patients to stop therapy[47].

ENDOSCOPIC TREATMENT

Several endoscopic techniques have been evaluated, however only argon plasma coagulation (APC) and formalin application have consistently proved to be effective for treating severe rectal bleeding. Other approaches, such as radiofrequency ablation (RFA), cryoablation and band ligation should not be considered of choice in the clinical setting (Table 2). ND: RAG laser treatment should be considered as an obsolete treatment, fully replaced by APC treatment.

Argon plasma coagulation

Rationale: Argon plasma coagulation is a noncontact technique with a governable depth of coagulation (0.5-3 mm), which applies a high-frequency current to the tissue and burns bleeding vessels, thus stopping rectal hemorrhage. As compared to ND: YAG laser therapy, APC is much more easier to use and safer; however, RCTs matching the two techniques have not been performed so far.

Evidences: The evidence supporting the employment of APC for treatment of clinically significant, intractable rectal bleeding cannot be judged as of high quality. Indeed, evidence comes from several retrospective and prospective case-series and observational studies, while only a few, small-sized RCTs comparing APC to formalin application have been conducted[49-58]. A systematic review focusing on studies published upon 2011 found that APC improved or completely resolved symptoms in 50% to 100% of patients[59]. Since then, a prospective observational study and an RCT have been published. Sato *et al*[60] performed a prospective observational study considering 65 patients with chronic rectal bleeding, and found that APC was successful in improving symptoms in 60 (94%) of them after a mean follow-up of 35 mo. Yeoh *et al*[61] randomized 30 patients with intractable rectal bleeding to receive APC or formalin endoscopic treatment, and concluded that APC was effective in treating symptoms in 94% of patients. Indeed, only one patient required further intervention after a follow-up of 111 mo.

Argon plasma coagulation should be considered as the treatment of choice when clinically significant bleeding occurs. As APC burns not only the bleeding vessels, but also mucosa and submucosa, it can lead to ulcerations, sometimes associated with chronic pain and slow healing[62]. Thus, APC should be performed reducing argon flow rates (≤ 2 L/min) and wattage (≤ 40 watt). Adverse events are mild in most cases, and have been

reported in up to 18% of patients[55]. Abdominal cramps are the most frequently described side effects, occurring due to the colonic distention induced by argon gas; thus, two-channel endoscopes should be employed in order to insufflate and contextually remove argon gas during the procedure. Ulcerations have been often reported too[62]. Severe complications have been rarely described, including gas explosion and perforation, fistula, stricture, and long-term pain[51,58,62]. Notably, colonic explosion mostly occurred when the endoscopic procedure was performed after inaccurate, local bowel cleansing with enemas, instead of gold-standard oral preparation[53,57].

Formalin

Rationale: Formalin is an aldehyde commonly used to preserve or fix tissues by cross-linkage of primary amino groups in proteins with other nearby nitrogen atoms in proteins or DNA through a CH2-linkage. As formalin is highly irritant to biologic tissues, when directly applied to radiation-damaged tissues it induces local chemical cauterization that scleroses and seals fragile neo-vasculature[63]. Thus, formalin has been proposed as a treatment for refractory severe rectal bleeding.

Evidences: Formalin might be considered as alternative to thermal coagulation therapy with argon plasma in patients with severe rectal bleeding. However, the existing evidence upon the role of formalin in PRD is not completely satisfactory: Indeed, three randomized controlled trials have been conducted so far, two of which are published in abstract form only[50,54,61]. Yeoh *et al*[61] randomized 30 patients suffering from severe rectal bleeding to receive either argon plasma coagulation or formalin application, and found that both treatments were not differently effective, as control of rectal bleeding was achieved in all patients.

Topical formalin therapy can be performed with an operating sigmoidoscope under general anesthesia. It is important to smear the anus and buttocks with petroleum jelly, in order to prevent direct contact with the formalin solution. Standard gauze pledgets soaked in 4% formalin solution have to be applied to the affected areas under direct vision, starting proximally. Each pledget needs to be held in place for 1 min for each affected area until all areas distally had been treated[61,64]. Endoscopic application of formalin is more frequently associated with complications and requires more skilled endoscopists than argon plasma coagulation therapy[65]. The most frequently reported adverse events include ano-rectal pain, fecal incontinence, severe diarrhea, fever and the severe formalin-induced colitis[66]. Other complications include anal or rectal strictures, rectal perforation or ulceration.

RFA

Rationale: RFA is an endoscopic procedure in which a target tissue is ablated using the heat generated from high frequency alternating current[67]. RFA, performed with the BARRx Halo90 system used to treat Barrett's esophagus, has been recently proposed for severe intractable rectal bleeding. In comparison with APC, RFA allows broader areas of tissues to be treated and inducess prompt squamous re-epithelialization with prevention of re-bleeding; furthermore, RFA is restricted to the superficial mucosa, thus it could represent a safer alternative to traditional endoscopic treatments[63].

Evidences: Up to now, the role of RFA as an alternative endoscopic treatment for severe intractable rectal bleeding has yet to be defined. Indeed, no randomized controlled trial has been performed, thus the quality of evidence supporting the use of RFA is poor[68-72]. Rustagi *et al*[72] performed the largest observational study concerning RFA technique in PRD. Thirty-nine patients were enclosed, and all of them experienced complete resolution of rectal bleeding during a mean follow-up of 28 mo. Furthermore, treatment with RFA led to discontinuation of blood transfusion and iron therapy in 92% and 82% of patients, respectively. As far as the existing, unsatisfactory evidence is concerned, RFA can be regarded as a relatively safe procedure[68-72]. Indeed, the most frequently reported side effects were mild-to-moderate anorectal pain, transient fecal incontinence, asymptomatic perianal ulceration and difficult evacuation of stool[70,72].

Cryoablation

Rationale: Cryoablation is a non-contact therapy that employs liquid nitrogen to apply extremely cold temperatures to a targeted area, resulting in tissue destruction. Effects are both immediate and delayed, due to the induction of ischemic necrosis.

Evidences: Up to now, the evidence supporting cryoablation as a therapeutic option for PRD is absolutely scanty. Indeed, only a few small-sized case-series have been reported[73-75]. Thus, cryoablation might not be considered as a feasible alternative to other established endoscopic treatments. The largest case series was enrolled by Hou *et al*[75] who treated with cryoablation ten patients with chronic hemorrhagic PRD and found it to significantly improve rectal bleeding. However, this was a non-powered case series pilot study, therefore these results, though attractive, are not sufficient to draw any firm conclusion. As cryoablation has not yet been performed in an adequately large sample of patient, it cannot be still considered as a safe procedure. In fact, the major risk associated with the procedure consists of colonic over-insufflation resulting in cecal perforation[75].

Rectal band ligation

A case report described the use of rectal band ligation in a patient with radiation-induced rectal bleeding not responsive to endoscopic conventional treatment, *i.e.*,

APC. Five bands were placed in two separate sessions, with nearly total eradication of rectal teleangiectasias and without complications[76]. Obviously, though encouraging this result is anecdotic, thus further studies are warranted to define the role of rectal band ligation for treating PRD.

CONCLUSION

The management of pelvic radiation disease may be challenging; several treatment options exist and the choice should be based on the best available evidences. Most of the studies have investigated the management of radiation-induced rectal bleeding. Patients with clinically significant bleeding (*i.e.*, causing chronic anemia) should firstly be considered for medical management (*i.e.*, sucralfate enemas, metronidazole and HBO), in case of failure, endoscopic treatment should be implemented. This latter should be considered the first choice in case of acute, transfusion requiring, bleeding. Alternative treatments, such as embolisation or surgery, should be considered in case of acute severe bleeding once endoscopy has failed. More well performed, high quality studies should be performed, especially the role of medical treatments should be better investigated as well as the comparative studies between endoscopic and HBO treatments.

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Cervical cancer screening in developing countries at a crossroad: Emerging technologies and policy choices

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Abstract

Cervical cancer (CC) represents the fourth most common malignancy affecting women all over the world and is the second most common in developing areas. In these areas, the burden from disease remains important because of the difficulty in implementing cytology-based screening programmes. The main obstacles inherent to these countries are poverty and a lack of healthcare infrastructures and trained practitioners. With the availability of new technologies, researchers have attempted to find new strategies that are adapted to low- and middle-income countries (LMIC) to promote early diagnosis of cervical pathology. Current evidence suggests that human papillomavirus (HPV) testing is more effective than cytology for CC screening. Therefore, highly sensitive tests have now been developed for primary screening. Rapid molecular methods for detecting HPV DNA have only recently been commercially available. This constitutes a milestone in CC screening in low-resource settings because it may help overcome the great majority of obstacles inherent to previous screening programmes. Despite several advantages, HPV-based screening has a low positive predictive value for CC, so that HPV-positive women need to be triaged with further testing to determine optimal management. Visual inspection tests, cytology and novel biomarkers are some options. In this review, we provide an overview of current and emerging screening approaches for CC. In particular, we discuss the challenge of implementing an efficient cervical screening adapted to LMIC and the opportunity to introduce primary HPV-based screening with the availability of point-of-care (POC) HPV testing. The most adapted screening strategy to LMIC is still a work in progress, but we have reasons to believe that POC HPV testing makes part of the future strategies in association with a triage test that still needs to be defined.

Key words: Low- and middle-income countries; Cervical cancer screening; Human papillomavirus testing

Core tip: Cervical cancer (CC) burden in developing countries remains important because of the difficulty in implementing cytology-based screening programmes. With the introduction of new technologies, researchers have attempted to find new strategies for CC screening adapted to these countries. Rapid human papillomavirus (HPV) tests are one of these advantageous methods. However, HPV testing has a low positive predictive value for CC, so a triage test is needed. Visual inspection tests, cytology and novel biomarkers are some options. We provide an overview of current and emerging screening approaches for CC. We discuss the challenge of implementing an efficient CC screening adapted to developing countries and the opportunity to introduce primary HPV-based screening with the availability of point-of-care tests.

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INTRODUCTION

The incidence of cervical cancer (CC) varies greatly worldwide. There is a large difference between developing and developed countries, where CC cases have been significantly reduced since the implementation of effective screening programmes. However, in developing countries, the burden from CC remains because of the difficulty in implementing cytology-based screening programmes. According to the latest world cancer statistics[1], CC is the fourth most common cancer in women globally (528000 new cases each year) and the second most common in developing areas (445000 new cases each year). CC is also the fourth most lethal cancer in women worldwide (266000 deaths) and the third cause of cancer-related death in developing countries (230158 deaths)[1], which means that more than 80% of the global burden occurs in developing areas.

In addition, the incidence and mortality of CC is variable within low- and middle-income countries (LMIC). In India, there are 20.2 per 100000 new cases of CC diagnosed and 11.1 per 100000 deaths annually, accounting for more than one fifth of the global CC deaths[2]. In sub-Saharan Africa, 34.8 per 100000 women are diagnosed with CC annually and 22.5 per 100000 women die from this disease[1]. In contrast, in western Asian countries, only 3.8 per 100000 new cases are diagnosed per year and 1.6 per 100000 die from CC[1]. Therefore, if the chances to survive CC are considered, a woman in Thailand will have an approximately 58% chance of survival, while in India she will only have a 42% chance. This survival is even more critical in Sub-Saharan Africa, where women only have a 21% chance to survive CC[3]. Overall, the mortality to incidence ratio of CC is 52%[4].

Human papillomavirus (HPV) is a major co-factor of CC. Development of vaccines against HPV has been a major advance for prevention of this cancer. Nevertheless, large-scale implementation of HPV vaccination is still lacking in developing countries and will not replace the need for CC screening.

In LMIC, there are several issues and challenges associated with CC screening. The main failure to implement an effective screening programme is related to the complexity of the screening process and the obstacles inherent in these countries. Poverty, limited access of the population to information, lack of knowledge of CC, the absence of sustained prevention programmes, lack of healthcare infrastructure required and lack of trained practitioners are the main obstacles to implementation of CC screening programmes[5]. Socio-religious and cultural barriers may also play an important role, as shown in an attempt to screen for CC in Peru[6]. Finally, government resources may be allocated to competing public health programmes with higher visibility and international attention than CC screening.

In this review, we discuss the challenge of implementing an efficient cervical screening adapted to LMIC and the opportunity to introduce a primary HPV-based screening with availability of a rapid HPV test.

ACTUAL SCENARIO AND DIFFICULTIES FOUND IN LOW-RESOURCE AREAS FOR CC SCREENING

At the present, very few developing countries have been able to implement CC screening programmes. To screen successfully in LMIC different requirements are important. The programme shall ensure wide coverage of the target population; it must guarantee screening, management and adequate follow-up of patients; it shall be provided on-site and be low-cost, with minimum infrastructure requirement that can lead to immediate treatment if abnormal. CC screening should be planned in line with other national programmes for cancer control. Moreover, in order to implement CC screening policies in these countries, a support and funding from the Ministry of Health is indispensable.

In the Middle East and North Africa, the first steps to implement national screening programmes based on visual inspection tests are being currently completed[7]. In contrast, in Sub-Saharan Africa, it is estimated that less than 5% of women at risk have ever been screened[8]. In India's case, guidelines for population-

Table 1 Obstacles to cervical cancer screening in low- and middle-income countries

Practical/logistical reasons
Widespread poverty
Lack of healthcare infrastructure
Absence of sustained prevention programmes
Lack of trained practitioners
Lack of laboratory supplies
Lack of patient management guidelines
Limited physical access of the population
Knowledge, religion and beliefs
Lack of knowledge of cervical cancer
Limited access of the population to information
Women disempowerment
Socio-religious and cultural barriers to routine pelvic screening
Political
Lack of support from the Ministry of Health
Competing healthcare priorities
War and civil strife
Others
High temperatures in tropical countries with lack of proper climatisation
Particularities about the screening test
VIA
Significant number of unnecessary and unsustainable treatment
Cytology
Need important health-care resource and infrastructure
Need important laboratory supplies
Screening requires more than one visit (important drop out)
Further testing with colposcopy wouldn't be possible, leading to unnecessary agressive and unsustainable treatment
HPV
Need important healthcare infrastructure
Need important laboratory supplies

VIA: Visual inspection tests with 3%-5% acetic acid; HPV: Human papillomavirus.

based screening programmes for cervical cancer are established for about 10 years[9] and are based on visual inspection tests. However, despite the introduction of these national guidelines, screening coverage is still very low[10]. Several obstacles are responsible for the failure to implement an effective screening program in LMIC. A summary of these obstacles is respresented in Table 1.

Cytology screening

Cytology screening (Pap test) for CC, especially as part of organised screening programmes, is the oldest and most widespread cancer screening technique. This technique has lead to effective reduction in the incidence and mortality from CC in many developed countries[11-13]. CC screening is one of the most successful disease-prevention programmes. However, this approach has failed to attain the same results in developing areas. A cytology-based screening programme requires repeat testing and visits to identify women who need treatment. Besides a cytopathologist, a colposcopy specialist and a pathologist should also be involved. To guarantee the success of a screening programme, training and continuing education are essential[14]. Previous experience has shown no decline in the incidence and/or mortality of CC, and this is probably because of low-quality cytology smears[8]. Consequently, implementation and execution of the whole process is too complex and expensive.

Moreover, even if implementing a high-quality cytology programme in these countries is possible, it would only be moderately effective. This is because the currently used Pap test misses approximately 50% of high-grade precursor lesions and cancers with a single screening[15]. Additionally, in low-resource settings, women would probably only be screened once or twice in their lifetime.

Visual inspection tests

Visual inspection tests with 3%-5% acetic acid (VIA) and/or Lugol's iodine (VILI) appear to be a satisfactory alternative screening approach to cytology. These tests have been used since the 1990s, mainly in poor resource settings. They are simple, cost-effective with relative ease of use[16-19], and may be performed by different healthcare workers (physicians, nurse, midwives and technicians). Moreover, this approach does not require high technology or infrastructure and has been shown to reduce mortality in developing countries[20,21]. The visible changes that occur in the cervix after application of acetic acid are immediate, and can be categorised as negative or positive for cervical neoplasia. These immediate results facilitate a same-day screen and management strategy. Therefore, this allows most of the eligible women to participate in the programme by minimising repeat visits. Evidence shows that this single-visit approach leads to the most significant decrease in high-grade cervical intraepithelial neoplasia (CIN)[22] and it is regarded safe, acceptable and fairly effective in India and Sub-Saharan Africa[17,23]. Despite the limitations of the concept of "screen and treat", it helps to overcome barriers of time, distance and loss to follow-up. This is relevant, because in a low-resource context, recalling patients for additional testing or treatment can be a critical component to a programme's success (Figure 1).

The performance of VIA has been evaluated in numerous studies[18,19,24-26]. An extensive meta-analysis by Sauvaget *et al*[19] pooled data from 26 studies that were conducted in different high- and low-income countries. They found an overall sensitivity of 80% and a specificity of 92% for the VIA method, although sensitivities greatly varied between studies. Close values were found in a meta-analysis where pooled data from 11 studies that were performed in Africa and India showed a sensitivity for VIA of 79% (range: 73%-85%) and a specificity of 85% (range: 81%-89%) for CIN2 lesions or worse (CIN2+)[18]. With regard to VILI, its use appears to increase VIA's sensitivity by 10%, without affecting the specificity[18,24,26].

VIA and VILI also have some drawbacks that need to be addressed. Interpretation of a visual test of the

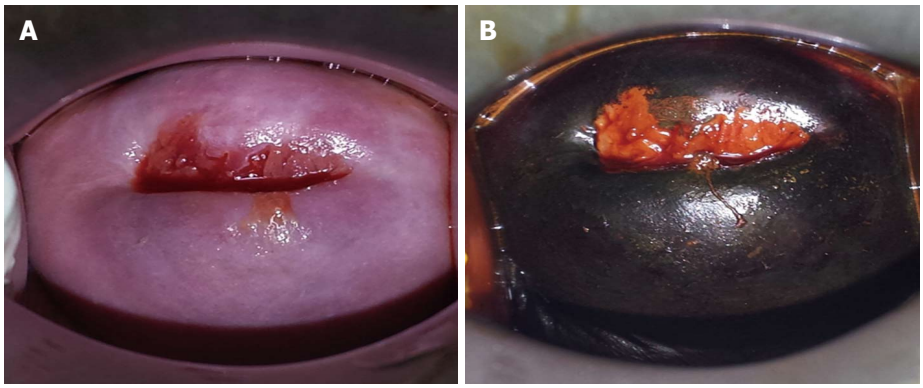

Figure 1 Visual inspection tests. A: Visual inspection test with 3%-5% acetic acid; B: Visual inspection test with Lugol's iodine.

cervix has limited value in older women because of degenerating cervical epithelium and partial or lack of visibility of the transition zone with ageing. Indeed, studies have shown that VIA sensitivity declines substantially in women aged 40 years or older[27,28]. VIA-based screening is also healthcare provider dependent and lacks reliable quality assurance control. As a consequence and to maintain high quality, implementation of VIA screening at primary and secondary facilities would require close supervision, which is difficult to attain at a national level.

More importantly, reported sensitivity for detecting CIN2+ widely varies in different studies (37%-96%), as does specificity (49%-98%)[27,29], which makes it dependent on the skill of the provider. Finally, studies that were conducted under screening conditions to assess the sensitivity and specificity of VIA used the gold standard of colposcopy, and this technique has been proven to yield error in the recognition of disease[30]. Because of these drawbacks, alternative methods need to be developed to improve, complement, or even replace VIA.

HPV TESTING FOR PRIMARY SCREENING

In recent years, there has been overwhelming evidence that HPV testing is more effective than cytology for CC screening, providing increased reassurance and allowing longer screening intervals to be adopted[31]. Highly sensitive tests have been developed and are currently used to replace cervical cytology for primary screening[32].

Currently, in the worldwide market, there are at least 150 different HPV tests available for the detection of alpha-HPVs and over 95 variants of the original tests. However, only some commercial HPV tests have documented clinical performance compared with the standard HPV test. According to guidelines, a candidate test should present a clinical sensitivity for CIN2+ of at least 90%, and a clinical specificity of at least 98% of that of the reference assays[33,34]. Regardless, the number of assays for HPV that have been approved by the Food and Drug Administration is increasing over time[33,35,36].

Moreover, among HPV tests, there is an important difference concerning the choices of primers to be used. Because of this overwhelming amount of choice available, choosing which HPV test is more suitable given a certain context can be difficult. Furthermore, and paradoxically, clinicians are generally not involved in choosing the HPV test.

Evidence shows that HPV tests should not only be type specific but also virial region specific (specific regions in the HPV genome are L1, E1/E2 and E6/E7). Indeed, during integration of HPV in the human genome, L1 expression is sometimes lost, but E6/E7 expression always remains present, which explains why there are not E6- or E7-negative cancers[37]. A test designed only for L1 will miss approximately 10% of all invasive cancers. This is why an HPV test is not recommended by some authors as a stand-alone test in CC screening programmes[37].

Current HPV tests are able to detect the presence of viral markers by signal amplification techniques, such as the Digene Hybrid Capture® Ⅱ assay or by amplification of nucleic acid with polymerase chain reaction. When combined with Pap smears, HPV tests can achieve nearly 100% sensitivity and a specificity of 93% in women aged 30 years and older, with a negative predictive value of almost 100%[38].

Several studies support that HPV testing is feasible in low-resource settings and appears to be the best strategy for CC in this context[17,24,39]. A large-cluster randomised trial from rural India showed that a single round of HPV screening could reduce the incidence and mortality from CC of approximately 50%, whereas approaches based on VIA and cytology had little effect on these outcomes[40].

Until recently, the greatest limitations of HPV testing were the need for expensive laboratory infrastructure and the 4-7 h time to process the test. The development of rapid molecular methods for detecting HPV DNA (*e.g.,* care HPV® - Qiagen, GeneXpert® - Cepheid) for screening or other POC type of tests is a milestone in CC screening in low-resource settings. This is because these new options may make screening more feasible in

the future and reduce the infrastructural requirements of previous screening programmes.

In a cohort of unscreened women aged 30 and over from South Africa, HPV testing followed by the treatment of HPV-positive women at the second visit was the most effective option (27% reduction in the incidence of CC) at a cost of 39 USD/years of life saved (YLS)[41]. VIA combined with the immediate treatment of women who tested positive at the first visit was cost saving and was the next most effective strategy, with a 26% decrease in the incidence of CC[41]. In another cost-effectiveness analysis in a rural Chinese population, where the careHPV® test (Qiagen, Gaithersburg, MD, United States) was directly compared with VIA, a once-per-lifetime screening at the age of 35 years would reduce CC mortality by 8% combined with VIA (cost of 557 USD/YLS), compared with 12% with the careHPV test (cost of 959 USD/YLS)[42].

Self-vaginal sampling for HPV testing

HPV - based screening requires that a sample be taken using a swab or brush by a healthcare provider or by the patient herself. The greatest advantage of HPV-based testing is obvious in that it allows sample collection to be performed by the patient herself, not requiring trained personnel and infrastructure to perform a pelvic examination. The criteria for a good quality sample are less rigorous with HPV testing compared with cytology. Many studies have shown that offering self-sampling for HPV testing (Self-HPV) can improve attendance to a CC screening programme and it is well accepted among women[39,43-45]. This strategy can not only be more appealing to non-attendees in developed countries, but also makes CC screening accessible to women in LMIC[46,47]. Evidence from multiple prospective studies has shown that the accuracy of Self-HPV versus clinician-collected specimens to detect precancerous lesion is comparable for the detection of precancerous and cancerous lesions[39,48,49]. Because of the numerous advantages of self-HPV, it will become a major focus of CC screening programmes worldwide in the near future.

TARGETED AGE FOR INITIAL SCREENING

The most relevant approach to identify women at risk for CC or pre-cancer is by age restriction. The World Health Organization (WHO) recommends targeting HPV screening to women who are 30 years of age and older because of their higher risk of CC, and that priority should be given to screening women aged 30-49 years (WHO screening recommendation update 2014). In addition, VIA is less effective in women aged older than 50 years because the squamocolumnar junction is less visible in menopausal women. If HPV is used as primary screening, recent evidence supports its use in women aged 30 years and over[50,51]. Most HPV infections are transient at an age younger than 30 years Therefore, the screening of young women leads to unrequired assessment and potentially to treatment of cervical lesions that might have regressed spontaneously[52,53]. However, even in women aged ≥ 30 years, most of HPV infections are transient, and only a small fraction of cases with persistent infection are at risk of CC[54]. Therefore, selecting HPV-positive women aged older than 30 years who are most likely to have or to develop a CC precursor in the future and require treatment is necessary for further evaluation (triage).

TRIAGE OF HPV-POSITIVE WOMEN

HPV-based screening has a low positive predictive value for CC because it does not directly test for cancer, but for HPV infection instead. A negative HPV test only indicates a low probability for the patient to develop CC within 5-10 years, and a positive result is only an indication of the presence of an essential risk factor. Therefore, women who test positive for HPV must be further evaluated to determine the optimal management. At the present time, three candidates can potentially be used as triage test: (1) visual methods (VIA/VILLI; (2) cytology; and (3) molecular testing. To date, there is no clear evidence to determine which strategy should be prioritised. Therefore, the choice of test essentially depends on the available health resource (Figure 2).

Triage with VIA/VILI

Triage with VIA/VILI offers the dual benefit of HPV screening to maximise detection of the disease and VIA/VILI for triage. In low-resource areas where the necessary equipment is lacking, VIA/VILI following an HPV-positive test is probably a good option, offering the possibility to adopt a "see and treat" approach. VIA/VILI will identify women with a precancerous change requiring immediate treatment by cryotherapy or cold coagulation, and those women in which cancer is suspected who should be referred to a specialised centre to receive aggressive multimodal treatment. Women with a negative VIA/VILI will be followed without treatment.

Triage with cytology

Triage with cytology is proposed in developing (middle income) countries where infrastructure exists with experience of screening[55]. However, healthcare providers should be aware that cytology is associated with multiple clinic visits and delays between screening, laboratory results, colposcopy and ultimately treatment, which are major barriers to the success of this method.

Triage with molecular tests

Cervical carcinogenesis is characterized by the integration of HPV DNA into the host cell genome resulting in abnormal proliferation of basal and parabasal cells due to the deregulated expression of viral oncoproteins, leading ultimately to the development of CC[56].

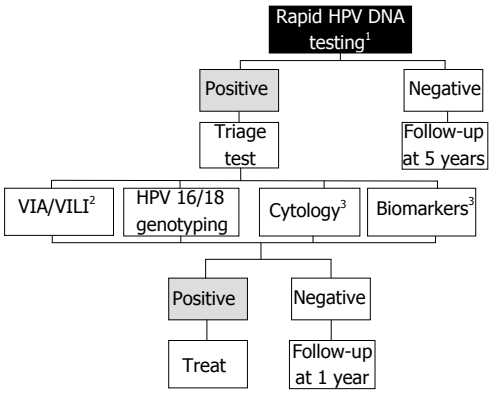

Figure 2 Decision making algorithm for human papillomavirus triage. [1]HPV testing done on a self-taken sample by women aged 30-50 years; [2]Triage tests suitable for same-day Screen and Treat; [3]Triage tests requiring a second visit for treatment. HPV: Human papillomavirus; VIA: Visual inspection test with 3%-5% acetic acid; VILI: Visual inspection test with Lugol's iodine.

Therefore, the detection of HPV DNA is used by many assays and is the only molecular marker fully developed and approved for primary CC screening. These tests can be based on the detection of specific types of oncogenic HPV that identify women at a higher cancer risk (*e.g.,* HPV genotypes 16 and 18)[36]. However, many other molecular mechanisms associated with HPV infection are necessary for CC development, such as chromosomal abnormalities, expression of oncogenes[57], epigenetic regulation (hypermethylation)[58] and apoptotic markers, which covers a large number of potential biomarkers. Molecular tests have been lately under intensive study as a potential alternative and triage tests for CC screening[59].

Expression of oncogenes: Oncoproteins expressing viral oncogenic activity could potentially be used as biomarkers in the triage of HPV-positive women or directly as a primary screening method. When HPV-infected cervical cells undergo precancerous or cancerous changes, oncoprotein E6 is expressed in cervical cells at elevated levels. Only E6 protein from high-risk HPV types promotes carcinogenesis by binding to a human PDZ domain. This allows E6 protein to bind to cellular molecules and deregulate cellular proliferation and differentiation, which may lead to the development of cancer[60]. An HPV E6 test using lateral flow (OncoE6™, Arbor Vita Corporation) has been developed to detect E6 protein of HPVs 16, 18 and 45[61]. Weaknesses of the OncoE6™ Cervical Test are low sensitivity (approximately 45%)[62] because it only detects HPV 16, 18 or 45. Additionally, specimens stored in buffers/transport medium used for HPV DNA testing cannot be used, and thus new cervical collection is always required. The oncogenic activity of E7 protein may also be tested indirectly by the host cyclin-dependent kinase inhibitor p16Ink4a. This kinase inhibitor decelerates the cell cycle by inactivating the cyclin-dependent kinases (CDK4/CDK6) involved in retinoblastoma protein phosphorylation[63]. Overexpression of p16INK4a in almost all cervical precancer (High-grade lesions) and invasive CC[64,65] has been shown to be directly linked to the transforming activity of E7 oncoprotein, which is produced by HPV[66]. Cellular accumulation of p16INK4a can be measured by cytochemistry using ELISA assays, which are commercially available (CINtec® p16, Roche mtm laboratories, Mannheim, Germany).

Modulation of host microRNAs and methylation status of protein-coding genes: HPVs modulate expression of host microRNAs (miRNAs)[67] *via* deletion, amplification, or genomic rearrangement. Recent studies have explored the role of the miRNAs in the development of CC. They found that several miRNAs are dysregulated in CC, such as miR-21, miR-127, miR-143, miR-145, miR-155, miR-203, miR-218 and miR-214, among others[68-72]. The miRNA-203 is downregulated in HPV-positive cells and its repression leads to maintenance of increased levels of p63 in infected suprabasal cells, maintaining cells in an active state in the cell cycle[73]. Other well studied miRNA is the miRNA-21, whose upregulation has been associated with aggressive progression and poor prognosis in CC[74]. Also miRNA-143 and -145 were found to be less expressed in CC[67,70]. Despite being a hot-spot topic, some discordance exists between studies concerning miRNAs, therefore further studies need to be conducted before these molecular biomarkers can be safely introduced in CC screening routine.

Epigenetic silencing of tumor suppressor genes is also responsible for cervical carcinogenesis[58]. Quantification of DNA methylation can be easily done and has been drawing attention in the recent years, making it a promising biomarker in CC[75]. L1 genes from HPV16 and 18 L1 are always highly methylated in CC[76,77]. A recent study using a rapid and sensitive technique[77], methylation-sensitive high-resolution melting analysis, has shown that L1 HPV16 methylation was highly associated with cervical pre-cancer and cancer and can be used as a triage test for women positive for HPV16 who are at greater risk to develop invasive cancer. Another study on HPV DNA methylation[78] tested 14 methylated candidate genes (ADRA1D, AJAP1, COL6A2, EDN3, EPO, HS3ST2, MAGI2, POU4F3, PTGDR, SOX8, SOX17, ST6GAL2, SYT9, and ZNF614) and found that POU4F3 gene methylation had the highest area under the ROC curve (0.86; 95%CI: 0.78-0.95) in detecting CIN3+, which makes it a potential molecular tool for triage in HPV-positive women.

Other protein biomarkers: Promising additional molecular markers for triage of HPV-positive women are molecular markers expressing aberrant S-phase induction (BD ProEx™ C reagent), including two proteins: Topoisomerase ⅡA and minichromosome maintenance protein. Both proteins are overexpressed in HPV-infected cells as a result of the uncontrolled activation of the gene transcription and are linked to severity of cervical lesions[79,80]. Moreover, carcinoma embryonic antigen

has found to be a good biomarker for CC prognosis and disease management[81], though it is elevated in different non-cancerous and cancerous conditions. Many other biomarkers, such as integral membrane protein CD44, enzyme cyclooxygenase-2, cytokine vascular endothelial growth factor and membrane protein caveolin-1 might be useful in CC screening, by being more or less associated with cervical lesions severity, disease progression and prognosis[82-85].

CONCLUSION

Emerging technology places CC screening in developing countries at a crossroad and a choice of new policies is warranted. Primary HPV testing is widely accepted as being more effective than cytology for CC screening. Primary HPV testing increases sensitivity for the detection of CIN2+ compared with cytology and its high negative predictive value allows screening intervals to be extended. However, HPV testing has a mediocre specificity and positive predictive value. Additionally, HPV testing could be impractical in developing countries without a triage strategy to further characterise and evaluate the risk of an HPV-positive woman. Therefore, follow-up and management should be carried out. The emergence of rapid POC HPV tests that are performed in self-obtained vaginal samples will permit not only first-line screening, but also a triage of HPV-positive women during the same visit. As a result, a new concept can be achieved in a single visit, consisting of self-HPV testing, triage and treatment. This could allow most of the eligible women living in low-resource settings to participate in a CC screening programme by minimising repeated visits.

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Neoadjuvant chemotherapy followed by surgery in gastric cancer patients with extensive lymph node metastasis

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Abstract

Gastric cancer with extensive lymph node metastasis (ELM) is usually considered unresectable and is associated with poor outcomes. Cases with clinical enlargement of the para-aortic lymph nodes and/or bulky lymph node enlargement around the celiac artery and its branches are generally dealt with as ELM. A standard treatment for gastric cancer with ELM has yet to be determined. Two phase Ⅱ studies of neoadjuvant chemotherapy followed by surgery showed that neoadjuvant chemotherapy with S-1 plus cisplatin followed by surgical resection with extended lymph node dissection could represent a treatment option for gastric cancer with ELM. However, many clinical questions remain unresolved, including the criteria for diagnosing ELM, optimal regime, number of courses and extent of lymph node dissection.

Key words: Extended lymph node metastasis; Gastric cancer; Neoadjuvant chemotherapy; Gastrectomy; Lymph node dissection

Core tip: Gastric cancer with extensive lymph node metastasis (ELM) is usually considered unresectable and associated with poor outcomes. Phase Ⅱ studies of neoadjuvant chemotherapy followed by surgery have shown the efficacy of this multimodal therapy for this pathology, but many clinical questions remain unresolved, including the criteria for diagnosing ELM, optimal regime, number of courses and extent of lymph node dissection.

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with extensive lymph node metastasis. *World J Clin Oncol* 2015; 6(6): 291-294 Available from: URL: http://www.wjgnet.com/2218-4333/full/v6/i6/291.htm DOI: http://dx.doi.org/10.5306/wjco.v6.i6.291

INTRODUCTION

Surgical resection represents the most important step in the treatment of gastric cancer and is the only approach offering complete cure. Systemic chemotherapy is administered for gastric cancer patients with liver, peritoneal, or other distant metastases, but extensive lymph node metastasis (ELM) stands on the borderline between surgical resection and systemic chemotherapy. The development of treatment for gastric cancer with ELM is a difficult and challenging task. This article reviews previous reports and looks to the future of treatment for gastric cancer with ELM.

ELM IN GASTRIC CANCER

No widely accepted consensus has been reached regarding the definition of ELM. In most reports, cases with clinical enlargement of the para-aortic lymph nodes (PAN) but no other distant metastases have generally been dealt with as ELM[1-3]. The range of PAN that are subject to surgical resection extends from the caudal end of the celiac axis to the cranial side of the inferior mesenteric artery, and is taken to be No. 16a2-b1 in the Japanese classification of gastric cancer[4].

Most reports consider positivity for PAN metastasis with enlargement of ≥ 1 cm in the long axis as ELM[1-3]. In the most recent response evaluation criteria in solid tumors (RECIST)[5], enlargement of ≥ 15 mm in the short axis is considered to represent measurable and assessable lymph nodes. Using multislice computed tomography, Marrelli *et al*[6] diagnosed clinical metastasis with enlargement of ≥ 8 mm in the short axis of PAN in gastric cancer, and reported positive and negative predictive values of 73% and 97%, respectively, with 93% accuracy.

Indications for surgical intervention do not always need to follow RECIST criteria, as the purpose of these criteria is to objectively evaluate treatment effect. However, further investigation of the criteria for diagnosing ELM is needed in the future.

In a series of Japan Clinical Oncology Group (JCOG) studies by Yoshikawa *et al*[3] and Tsuburaya *et al*[1], bulky lymph node enlargement around the celiac artery and its branches (bulky N), in addition to clinical enlargement of PAN, is treated collectively as ELM. The reasons are that patients with such metastases are commonly considered to be inoperable and, similar to PAN-positive patients, prognosis is poor even if curative resection is possible. According to the report by Tsuburaya *et al*[1], survival outcomes are equivalent in PAN-only patients and bulky N-only patients. Treating PAN metastasis and bulky N as a single disease group has thus been considered reasonable.

STANDARD TREATMENT FOR GASTRIC CANCER WITH ELM

From the 1980s to 1990s, PAN dissection was actively performed at some institutions in Japan. As a result, PAN metastasis was seen in about 20% of patients at maximum, with long-term survival achieved in about 10%-20% of these patients[7,8]. Similar reports have recently been seen from Western countries[9].

On the other hand, JCOG 9501 was conducted to verify the significance of prophylactic PAN dissection, but no meaningful impact was found[10]. As a result, while PAN were categorized as regional lymph nodes in past Japanese classifications[4], the new classifications categorize them as distant lymph nodes[11] that are no longer considered a target of curative resection. PAN are also taken to be distant lymph nodes in Western guidelines[12], and are again not considered a target of curative resection.

However, it must be noted that the results of JCOG 9501 show the ineffectiveness of prophylactic PAN dissection. In that sense, a standard treatment for gastric cancer with clinical PAN metastasis has yet to be determined.

No comprehensive investigations of gastric cancer with bulky N have been reported. In some cases, curative resection may be achieved, but many cases are judged as unresectable because of direct invasion of major blood vessels.

TREATMENT OUTCOMES OF NEOADJUVANT CHEMOTHERAPY FOR GASTRIC CANCER WITH ELM

The JCOG 0001 was a phase Ⅱ clinical study for gastric cancer patients with ELM[3]. After excluding micrometastases to the liver and peritoneum by staging laparoscopy, irinotecan plus cisplatin combination therapy (IP) was administered as neoadjuvant chemotherapy. This was followed by gastrectomy with extended lymph node dissection including PAN. As a result of three treatment-related deaths, the study was discontinued. However, a subsequent follow-up and survival analysis showed a median survival time (MST) of 14.6 mo and a 3-year survival rate of 27% (95%CI: 15.2%-38.8%), exceeding the 3-year survival rate threshold (15%) established in the initial protocol. Although careful management of adverse events and appropriate patient selection are essential, this treatment could be recommended for gastric cancer patients with ELM.

In the JCOG 0405 study[1], S-1 plus cisplatin combination therapy (SP) was used for neoadjuvant chemotherapy in similar patients, and the primary

endpoint was the percentage of complete resections with clear margins in the primary tumor (R0 resection). Fifty-three patients were enrolled, and among the 51 who proved eligible, R0 resection was performed in 42 patients (82.4%). A subsequent survival analysis showed an unexpectedly good 3-year survival rate of 58.5% (95%CI: 44.1%-70.4%).

Some reports have shown better survival results in gastric cancer patients who had only abdominal lymph node metastases. Yoshida *et al*[13], investigated cases of long-term survival in patients who underwent chemotherapy for advanced gastric cancer, and reported that the 2- and 5-year survival rates of patients with metastasis to the abdominal lymph nodes only were 14.3% and 10.4%, respectively. Park *et al*[14] reported a 3-year survival rate of 13.1% in gastric cancer patients with isolated involvement of PAN.

In those reports, clinically metastases to the liver and peritoneum that were not obvious may not have been excluded, as such metastases could not be excluded with laparotomy or staging laparoscopy. In addition, the extent of abdominal lymph node involvement in those studies may have partly exceeded that of the two neoadjuvant studies. Accordingly, direct comparison of survival rates between these chemotherapy and neoadjuvant studies is inappropriate. Despite this, the 3-year survival rate seen in the JCOG 0405 study is notably high. Furthermore, Yoshida *et al*[13] and Park *et al*[14] reported that some kind of local therapy had been used in many cases of long-term survival in their articles. In view of these results, neoadjuvant chemotherapy followed by surgical resection could represent a useful treatment option for gastric cancer with ELM. However, Tsuburaya *et al*[1] described a lower 5-year survival rate for patients with both bulky N and PAN. The indication of neoadjuvant chemotherapy followed by surgery for this target is controversial.

OPTIMAL CHEMOTHERAPY REGIME FOR GASTRIC CANCER WITH ELM

From the results of the above-mentioned JCOG 0001 and JCOG 0405 studies, 2 or 3 courses of SP is currently recommended for gastric cancer patients with ELM. For unresectable or recurrent gastric cancer, on the other hand, several triplet regimes such as docetaxel/cisplatin/5-fluorouracil[15] or docetaxel/cisplatin/S-1 combination therapy (DCS)[16-19], have been developed and are reported to provide markedly high response rates. The JCOG 1002 study was therefore undertaken with DCS as neoadjuvant chemotherapy[20], and the results are scheduled to be published soon.

The optimal number of cycles for neoadjuvant chemotherapy has not been established, but 2 or 3 cycles of therapy have been adopted in most neoadjuvant studies. The COMPASS-D trial, a randomized phase Ⅱ trial with a factorial design comparing 2 and 4 courses of SP and DCS in neoadjuvant chemotherapy, is underway for curable gastric cancer with serosal invasion[21]. Informative results are expected in terms of optimal regime and number of courses of neoadjuvant chemotherapy for gastric cancer from that trial.

No detailed reports have been published regarding the optimal interval between neoadjuvant chemotherapy and surgery, but patients ordinarily receive surgery if they meet adequate organ functions according to laboratory testing within 14 d before surgery[20].

SIGNIFICANCE OF EXTENDED DISSECTION FOR GASTRIC CANCER WITH ELM

In the above-mentioned JCOG 0001 and JCOG 0405 studies, gastrectomy with D2 plus PAN dissection was performed following neoadjuvant chemotherapy. This strategy is based on the high rate of PAN metastasis seen not only in patients with clinical PAN metastasis, but also in patients with bulky N. In addition, complete elimination of cancer cells can hardly be expected with a few courses of neoadjuvant chemotherapy. Inoue *et al*[22] evaluated the efficacy and feasibility of neoadjuvant chemotherapy with SP in initially unresectable locally advanced gastric cancer, and reported 3-year survival rates of 31.0% and 53.8% in all and curative cases, respectively. However, they also reported that the most common site for initial recurrence after R0 resection was the PAN. Wang *et al*[2] reported an MST of 29.8 mo after performing gastrectomy with D2 dissection following capecitabine plus oxaliplatin combination therapy in patients with clinical PAN metastasis. Those results should be interpreted with caution, since selection bias for curative cases may have had an effect. The good survival outcomes in the JCOG 0405 and JCOG 0001 studies were obtained with PAN dissection as well as gastrectomy plus D2 lymph node dissection. Given these findings, gastric cancer with ELM should be treated using concurrent PAN dissection not only in patients with PAN metastasis, but also in bulky N-only patients. However, further investigation is needed regarding the optimal extent of lymph node dissection for gastric cancer patients with ELM.

CONCLUSION

A certain level of outcome is expected with multimodal therapy combining neoadjuvant chemotherapy and extended lymph node dissection in gastric cancer patients with ELM. At the same time, many questions remain to be unresolved, including the criteria for diagnosing ELM, optimal regime, number of courses and range of lymph node dissection.

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Carcinoma of unknown primary and paraneoplastic dermatomyositis

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Abstract

Dermatomyositis is known to be associated with neoplastic disorders, however the presentation of carcinoma of unknown primary as dermatomyositis is rare. We describe a case index of 50-year-old female who presented with enlarged inguinal lymph nodes accompanied with symmetric proximal muscle weakness and erythematous plaques. Conventional basic work-up did not reveal the diagnosis, however, positron emission tomography-computed tomography and re-staining of the pathology specimen suggested the ovaries as the primary site. Chemotherapy including carboplatin paclitaxel and bevacizumab led to complete response of disease and improvement in the dermatomyositis. The present case emphasizes the importance of a thorough directed evaluation for the underlying cancer in patients with carcinoma of unknown primary presenting as dermatomyositis. We further provide an up-to-date detailed review of published data describing these clinical entities.

Key words: Paraneoplastic; Dermatomyositis; Cancers of unknown primary; Positron emission tomography

Core tip: The presentation of carcinoma of unknown primary as dermatomyositis is rare. Positron emission tomography-computed tomography and pathology case oriented evaluation may identify the site of origin. We provide an up-to-date detailed review of published data describing these clinical entities.

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INTRODUCTION

Cancer of unknown primary origin (CUP) is a group of metastatic tumors for which the site of origin cannot be detected at the time of diagnosis. In most of the cases, the source of the cancer will never be determined.

According to the European society of medical oncology, CUPs account for up to 5% of all malignancies[1]. The biology of these tumors is not fully elucidated although mechanism of metastatic spread in the absence of growth of the primary tumor can occur through site-specific transformation of disseminated cells, or oncogene induction at metastatic stroma.

Dermatomyositis is a connective-tissue disease characterized by progressive, proximal muscle weakness and pathognomonic cutaneous findings. Malignancy is associated with dermatomyositis in up to 40% of patients, representing a paraneoplastic phenomenon[2,3]. We describe here a rare case of a female who presented with carcinoma of unknown origin accompanied with symmetric proximal muscle weakness and erythematous plaques.

CASE DESCRIPTION

A 50-year-old woman with carcinoma of unknown origin was admitted to the E.R because of intense weakness. She was evaluated one month earlier when she underwent biopsy from enlarged left inguinal lymph node. The biopsy revealed poorly differentiated carcinoma of unknown origin (performed out of our institute). On arrival, her physical examination revealed proximal weakness, which was profound in all extremities. Skin manifestations included periorbital edema and erythematous plaques on the extremities. She had enlarged left inguinal lymph node and signs of biopsy from the right lymph node. Biochemical analysis demonstrated elevated creatine kinase 5600 u/L (normal range 26-192), elevated AST 147(normal range 0-40) and ALT 130 (normal range 0-35). A computed tomography (CT) scan was unremarkable except enlarged left inguinal lymph node. Indirect immunofluorescence for anti-Jo-1 and ANA were negative. Tumor markers showed CEA-4.16 (0-4) CA15-3 -60 (0-30) CA125 80 (0-30). The patient underwent another biopsy from the left lymph node for re-pathology evaluation and staining which showed poorly differentiated Carcinoma. Staining for keratins: CK7 - positive strong, CK20 - weak, CK5/6 - negative, WT1 - positive and TTF - negative. Gynecological evaluation, colonscopy and endoscopy where unremarkable. Since dermatomyositis is associated with gynecological - ovarian cancer there was a clinical decision to look for findings at the urogenital system. Gynecologist examination and vaginal US were unremarkable. It was then decided to perform positron emission tomography (PET)-CT scan which demonstrated increased standardized uptake values uptake in the left pelvis in an ovarian cyst and there was also high standardized uptake values uptake in paraaortic and cervival lymph node (Figure 1). These observations have led us to re-staining the specimen for CA125 and p53 which were both positive suggestive of ovarian origin. The patient begun chemotherapy treatment with protocol directed to ovarian cancer including carboplatin [Area under the curve (AUC) 6] paclitaxel 175 mg/m^2 and bevacsizumab 15 mg/kg every three weeks. After 2 cycles dose reductions in both carboplatin (to AUC 4-5) and paclitaxel (to 80 mg/m^2 weekly) were needed due to neuropathy and neutropenia. She also received steroids (prednisone 60 mg which was gradually tapered off and replaced with methotrexate) for dermatomyositis. This treatment (chemotherapy and/or the steroids) led to complete response in the disease and improvement in the dermatomyositis.

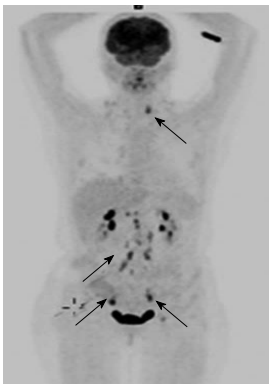

Figure 1 Positron emission tomography-computed tomography scan which demonstrated increased standardized uptake values uptake in the pelvis in an ovarian cyst and uptake in para-aortic and cervical lymph node (arrows).

LITERATURE REVIEW

Few studies demonstrated that dermatomyositis and polymyositis are association with different cancers[4-6]. A pooled analysis from Scandinavian repositories, confirmed that both dermatomyositis and polymyositis are associated with malignancy (dermatomyositis more than polymyositis)[3]. This study confirmed that ovarian, lung, gastric, colorectal, and pancreatic cancers were the cancers most strongly associated with dermatomyositis but other cancers were associated with dermatomyositis as well. Cancer treatment, local (surgery) or systemic (chemotherapy) usually results in remission of the dermatomyositis, and recurrence of symptoms can represent relapse of the malignant disease, further supporting its paraneoplastic origin[7,8]. Due to the increased risk of some cancers in patients with dermatomyositis, further examinations which may include whole body imaging, mammography and gynaecological evaluation are justified.

CUPs diagnosis requires pathology evaluation[1]. CUPs are categorized by pathological evaluation into: Differentiated carcinomas (well, moderately, or poorly); undifferentiated neoplasms; squamous cell carcinomas and carcinomas with neuroendocrine differentiation. In

the past CUP was characterized as an individual entity with dismal prognosis, while as our understanding of cancer biology evolved it became clear that CUP may retain the underpinnings of the primary origin[9]. Staining for keratins, may mislead the clinicians if interpreted incorrectly. For example our patients was CK7 positive and CK20 - weak - if weak is interpreted as negative, lung breast and thyroid cancers are more suspected. If CK20 - weak staining is interpreted as positive ovarian and pancreas cancers are suspected and WT-1, p53 and CA125 may add information as shown in our case. Full physical examination, blood and biochemistry analysis, and CT scans of thorax, abdomen and pelvis constitute the basic work-up in CUPs. Other evaluation should be only clinically guided. The minority of patients with CUP (less than 20%) belong to subsets with more favorable outcomes and treatment response and it is therefore crucial to identify this patients during the work-up[1,10,11]. Peritoneal carcinomatosis in females represent one example of this favorable risk CUP. Our patient pathology did not reveal serous papillary but rather poorly differentiated carcinoma. Moreover the disease distribution according the PET-CT was not classic for ovarian caner except uptake in the left ovary. However the decision to treat her with chemotherapy used for ovarian cancers seems rational.

PET-CT is one of the most sensitive imaging techniques to detect malignant lesions and has been used in different cancer conditions with quite success[12-15]. Selva-O'Callaghan *et al*[16] prospectively evaluated the role of PET-CT in the diagnosis of occult tumors in patients with dermatomyositis/polymyositis. Using PET-CT they evaluated prospectively 55 patients with myositis. 9 out of the 55 patients were diagnosed with cancer. PET-CT identified 6 out of the 9 patients. The authors concluded that PET-CT for diagnosing CUP in patients with myositis was an option comparable to other multi diagnostic tests.

As the radiotracer doses administered using PET-CT are relatively small, the risk is very low compared with the potential benefits. There are no known long-term adverse effects from such low-dose exposure and the potential benefits from PET-CT outweighs the risks which may include allergic reactions (rare and mild), and exposure to low amount of radiation[17].

CONCLUSION

In conclusion this rare case of patient with carcinoma of unknown primary emphasizes the importance of a thorough directed evaluation and the usage of PET-CT for the underlying cancer in patients with carcinoma of unknown primary presenting with dermatomyositis.

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ORIGINAL ARTICLE

Basic Study

Fluoxetine induces cytotoxic endoplasmic reticulum stress and autophagy in triple negative breast cancer

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Author contributions: Bowie M, Pilie P, and Ibarra Drendall C designed the experiments; Bowie M and Ibarra Drendall C performed most of the experiments and analyzed the data; Pilie P, Seewaldt V, and Yu D conceived the project; Wulfkuhle J and Petricoin E performed the protein microarray analysis; Lem S, Desai S, and Carter A helped with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and Western blotting; Hoffman A and Ambrose A helped with the cell lysates preparation; Ibarra Drendall C conceived and wrote the manuscript.

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Abstract

AIM: To investigate the mechanism of action of lipophilic antidepressant fluoxetine (FLX) in representative molecular subtypes of breast cancer.

METHODS: The anti-proliferative effects and mechanistic action of FLX in triple-negative (SUM149PT) and luminal (T47D and Au565) cancer cells and non-transformed MCF10A were investigated. Reverse phase protein microarray (RPPM) was performed with and without 10 μmol/L FLX for 24 and 48 h to determine which proteins are significantly changed. Viability and cell cycle analysis were also performed to determine drug effects on cell growth. Western blotting was used to confirm the change in protein expression examined

by RPPM or pursue other signaling proteins.

RESULTS: The FLX-induced cell growth inhibition in all cell lines was concentration- and time-dependent but less pronounced in early passage MCF10A. In comparison to the other lines, cell growth reduction in SUM149PT coincided with significant induction of endoplasmic reticulum (ER) stress and autophagy after 24 and 48 h of 10 μmol/L FLX, resulting in decreased translation of proteins along the receptor tyrosine kinase/Akt/mammalian target of rapamycin pathways. The increase in autophagy marker, cleaved microtubule-associated protein 1 light chain 3, in SUM149PT after 24 h of FLX was likely due to increased metabolic demands of rapidly dividing cells and ER stress. Consequently, the unfolded protein response mediated by double-stranded RNA-dependent protein kinase-like ER kinase resulted in inhibition of protein synthesis, growth arrest at the G1 phase, autophagy, and caspase-7-mediated cell death.

CONCLUSION: Our study suggests a new role for FLX as an inducer of ER stress and autophagy, resulting in death of aggressive triple negative breast cancer SUM149PT.

Key words: Inflammatory breast cancer; Endoplasmic reticulum stress; Autophagy; Apoptosis; Fluoxetine

Core tip: Our study demonstrates for the first time the complex but selective actions of Food and Drug Administration-approved, well-tolerated antidepressant drug known as fluoxetine (FLX) in malignant triple negative breast cancer (TNBC) cells. The significant reduction in cell growth of inflammatory TNBC line SUM149PT was a consequence of unfolded protein response induced by FLX and subsequent induction of autophagy and mitochondrial apoptosis, demonstrating the intricate crosstalk between endoplasmic reticulum and mitochondria in response to cellular stress. Combination of low dose FLX with existing regimen for TNBC may provide dual benefit of alleviating psychological distress, including depression and anxiety, and inducing death in aggressive tumor cells.

Bowie M, Pilie P, Wulfkuhle J, Lem S, Hoffman A, Desai S, Petricoin E, Carter A, Ambrose A, Seewaldt V, Yu D, Ibarra Drendall C. Fluoxetine induces cytotoxic endoplasmic reticulum stress and autophagy in triple negative breast cancer. *World J Clin Oncol* 2015; 6(6): 299-311 Available from: URL: http://www.wjgnet.com/2218-4333/full/v6/i6/299.htm DOI: http://dx.doi.org/10.5306/wjco.v6.i6.299

INTRODUCTION

A major roadblock to effective breast cancer therapy is development of de novo or acquired resistance. Triple-negative breast cancer, which lacks the expression of steroid estrogen and progesterone receptors as well as overexpressed HER2, accounts for 15%-20% of all breast cancers. Majority of triple negative breast cancers (TNBCs) are basal-like, among the most aggressive types, likely to develop chemotherapy resistance, and lack suitable targeted therapeutics[1]. Resistance to apoptosis is often the mechanism by which these cancers evade death. Thus, an alternative approach to trigger cell death is greatly needed.

Autophagy is an example of alternative mechanism of cell death. However, this evolutionarily conserved process in response to metabolic stress typically leads to cell survival. Autophagy is a process in which damaged or long-lived proteins and organelles are encapsulated in double-membraned vesicles called autophagosomes, targeted for lysosomal degradation, and released into the cytosol as intermediate metabolites for nutrient recycling and ATP production[2]. While evidence has been limited, autophagic cell death has been shown in cells with deficient apoptotic proteins[3,4], upregulated mitochondrial cell death protein BNIP3[5], and deficient tumor suppressor Von Hippel-Lindau[6]. The pro-death function of autophagy is believed to be due to prolonged digestion of cellular components or selective digestion of survival (over death) factors.

NFκB regulates diverse cellular processes in response to numerous stimuli, including unfolded protein response (UPR) as a result of oxidative and metabolic stress[7,8]. UPR is induced when there is a buildup of unfolded, misfolded or damaged proteins within the endoplasmic reticulum [*i.e.,* endoplasmic reticulum (ER) stress]. The goal of UPR is to stop general protein synthesis but allow selective synthesis of ER chaperones, such as binding immunoglobulin protein (BiP), to restore balance[9]. ER stress can directly induce autophagy through upregulation of BiP, which is required for autophagosome formation[10]. The repressive effect of BiP on UPR signal transducers, such as double-stranded RNA-dependent protein kinase-like ER kinase (PERK), inositol-requiring enzyme 1 alpha and activating transcription factor 6, is released during ER stress[11]. If proper protein folding capacity is not restored, then all three arms of UPR induce CCAAT/enhancer binding protein-homologous protein (CHOP) and growth arrest and DNA damage 34 (GADD34) to stimulate apoptosis. In some situations, autophagy is induced to promote cell survival by removal of accumulated ubiquitinylated proteins and aggregates[12]. Together, these studies demonstrate the integration of signals from autophagy, ER stress/UPR, and apoptosis in regulating cell survival or cell death.

The anti-cancer properties of widely used antidepressants, specifically the selective serotonin reuptake inhibitors (SSRIs), have received attention in the last two decades. Fluoxetine (FLX) was the first approved SSRI for depression, and it is still used today across a diverse population, including in many cancer patients, for the treatment of anxiety and/or depression. FLX is a well-tolerated drug with a mild side effect profile,

safe in overdose, and almost no associated withdrawal symptoms, even when compared to other SSRIs[13]. Like most SSRIs, FLX blocks the reuptake of serotonin (5-HT) at the pre-synaptic membrane, enhancing the actions of 5-HT on serotonin receptors at the post-synaptic neuron[14]. But FLX is known to have various off-target interactions, resulting in modulation of cancer cell growth. For example, a single study in rodents suggested that FLX stimulates malignant cell growth[15]. However, multiple epidemiological studies have shown no association between SSRI use and breast cancer risk[16,17]. Previous studies have shown FLX-induced cell death in a variety of malignant cell lines, including those originating from the prostate, colon, lung, ovary, breast, brain, and the immune system[3,18-24]. One study has implicated the inhibition of the extracellular signal regulated kinase 1 and 2 (ERK1/2) as a potential consequence of FLX's anti-tumor effect[20]. However, the exact role of FLX in modulating ERK1/2 pathway in breast cancer subtypes is currently unknown.

In this study, distinct molecular subtypes of breast-derived cell lines, including triple-negative (SUM149PT) and luminal (T47D and Au565) breast cancer cells as well as non-transformed MCF10A, were evaluated for their response to FLX exposure in regards to protein expression of key components of signaling pathways that mediate cell growth, survival and death. Our study demonstrates that the cell growth inhibition in rapidly dividing TNBC SUM149PT is due to ER and metabolic stress that leads to decreased translation of proteins along the RTK/Akt/mTOR and MEK/ERK pathways. Excessive ER stress and autophagy induced by FLX in SUM149PT eventually leads to cell death mediated by executioner caspase-7. Given this proposed anti-proliferative mechanism and safety profile, FLX may prove an ideal part of a targeted regimen against TNBC in future *in vivo* and clinical studies.

MATERIALS AND METHODS

Reagents

Tissue cell culture media, FBS, horse serum, and 2X Tris-glycine SDS loading buffer were obtained from Life Technologies. Insulin, hydrocortisone, epidermal growth factor (EGF), FLX, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and bovine serum albumin were from Sigma. Cholera toxin was obtained from Calbiochem. Mammary epithelial growth medium bullet kit was purchased from Lonza. Tissue protein extraction reagent (T-PER), bicinchoninic acid (BCA) assay, and SuperSignal West Dura chemiluminescent substrate were from Thermo Fisher Scientific. The complete protease and phosphatase (PhosSTOP) inhibitors were obtained from Roche Applied Science. Primary antibodies for Western blotting were as follows: LC3B (ab48394) from Abcam; ERK1/2 T202/Y204 (4370), AMPKα T172 (2535), p70 S6 Kinase (p70 S6K) T389 (9234), LC3B (3868), BiP (3177), PERK (5683), eIF2α Ser-51 (3398), PARP (9542), μ-Calpain (2556) from Cell Signaling Technology; β-actin (sc-47778), GADD34 (sc-8327), GADD153/CHOP (sc-575) from Santa Cruz Biotechnology; caspase-12 (PRS3195) from Sigma-Aldrich.

Cell culture

Most breast cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). T47D cells were cultured in alpha MEM prepared as previously described[25]. SUM149PT cells were originally obtained from Dr. Stephen Ethier (Karmanos Cancer Institute, Detroit, MI) and are commercially available (Asterand, Detroit, MI). SUM149PT cells were maintained in Ham's F12 supplemented with 5% FBS, 5 mg/mL insulin, and 1 mg/mL hydrocortisone. Au565, BT474, and lapatinib-resistant BT474 (R-BT474) cell lines were maintained in RPMI 1640 supplemented with 10% FBS and 2 mmol/L L-glutamine. R-BT474 cell line was kindly provided by Dr. Neil Spector (Duke University Medical Center, Durham, NC). MCF10A lines were cultured in two different media. MCF10A late passage cells were maintained in HuMEC complete media, while MCF10A early passage cells (generous gift of Dr. David Beebe, University of Wisconsin, Madison, WI) were grown in DMEM-F12 supplemented with 5% horse serum, 20 ng/mL EGF, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μg/mL insulin. Normal human mammary epithelial cells (HMEC) were obtained from Lonza and grown in HuMEC complete media. DKAT cell line is unique to our laboratory and maintained in supplemented MEBM[26]. All cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37 ℃ and have been authenticated by DNA fingerprinting at the Duke University Cell Culture Facility.

Reverse phase protein microarray analysis

The aforementioned cell lines were grown in 100 mm dishes for 24 h, followed by addition of FLX at a final concentration of 10 μmol/L and cell harvest after 24 h and 48 h of treatments. The indicated FLX concentration was previously tested in another breast cancer cell line[22] and served as a starting point for our proteomic study. Untreated (control) cells were run in parallel. This experiment was performed at least three different times Briefly, adherent cells were washed twice in cold 1 × PBS and lysed directly in dishes on ice with modified T-PER buffer as previously described[27]. Following centrifugation at 3000 g for 5 min at 4 ℃, each supernatant was transferred to clean microcentrifuge tubes. After determining the total protein content by BCA protein assay, samples were diluted in 2 × Tris-glycine SDS sample buffer with 2.5% 2-mercaptoethanol up to 2 mg/mL and boiled for 8 min. Samples were spun briefly and then stored at -80 ℃ until they were shipped in dry ice to George Mason University where subsequent lysate printing in triplicate, immunostaining, and reverse phase protein microarray (RPPM) analysis were performed. For this study, we examined the expression of 79 phosphorylated, total, and cleaved proteins that are thought to play a role in breast cancer cell proliferation,

survival, apoptosis, and metastasis. Enumerated are some antibodies used in the experiments: Akt S473 (9271), ERK1/2 T202/Y204 (9101), GSK-3α/β S21/S9 (9331), AMPKβ1 S108 (4181), mTOR S2448 (2971), p70 S6K T389 (9205), eukaryotic translation initiation factor 4G (eIF4G) S1108 (2441), NFκB p65 S536 (3031), Bax (2772), Bcl-2 S70 (2827), cleaved Casp-7 D198 (9491), cleaved Casp-3 D175 (9661), E-cadherin (4065), Vimentin (3295), Snail (4719), and SAPK/JNK T183/Y185 (9251) from Cell Signaling Technology; GSK-3α/β Y279/Y216 (44-604) from BioSource; IκBα S32/S36 (551818) from BD.

MTT assay

Cell viability or growth was measured by the MTT assay. Cells were seeded in triplicate at 1.8 × 10^4 per well in 1 mL complete media in 12-well plates and grown at 37 ℃ for 24 h. Subsequently, 10 μmol/L FLX was added in the cell media and incubated at the indicated time points. Day 0 reading was done at the same time as treatment was added. The MTT assay was carried out as follows: MTT solution was added at a final concentration of 0.5 mg/mL and incubated in the dark at 37 ℃ for 2 h. The reaction was stopped by adding Solubilization solution (95% DMSO/5% 1 × PBS), and absorbance values were determined at 560 nm on the Modulus microplate reader (Turner Biosystems). All MTT assays were performed at least two independent times.

Western blotting

Cell lines were seeded in 100 mm dishes at 2.6 × 10^5, followed by treatment with and without fluoxetine after 24 h. Cells were grown at the indicated FLX concentration and time points, harvested and lysed in radio-immmunoprecipitation assay buffer[28] containing phosphatase and protease inhibitor cocktails, and centrifuged at 14000 rpm for 10 min. The resulting supernatants (whole cell lysates) were assayed *via* BCA to determine total protein content and stored at -80 ℃ until use. Cell lysates were solubilized in reducing sample buffer, boiled, electrophoresed on Bis-Tris gel (Life Technologies), and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) The membranes were blocked and incubated with primary antibodies overnight at 4 ℃, washed 3 × in TBS with 0.1% Tween 20, incubated with horseradish peroxidase-conjugated secondary antibody, and detected by enhanced chemiluminescence.

Cell cycle analysis

Cells were seeded at 2.6 × 10^5 in 100 mm dishes. After 24 h, FLX was added at a final concentration of 10 μmol/L. Cells were harvested at indicated time points after treatment. Briefly, floating cells were retained and combined with trypsinized cells. Cells were spun down, washed with 1 × PBS, fixed in ice-cold 70% ethanol. Propidium iodide (PI) staining was performed as follows. Briefly, ethanol was removed and the cell pellets were resuspended in 1 × PBS containing 15-25 μg RNase A (Roche) and incubated for 30 min at 37 ℃. PI stain (2 mg/mL) was added to each sample at a final concentration of 100 μg/mL. Cells were sorted on a BD FACSCalibur using CellQuest software.

Statistical analysis

Data were analyzed with SAS Enterprise Guide 5.1 software (Cary, NC) and represented as the mean ± standard error. Two-sample *t*-test was used when comparing control and treated groups. To identify which proteins were differentially expressed in cell lines as a result of FLX treatment, changes in protein levels due to treatment were evaluated as percentages relative to control *via* 1-way ANOVA with Tukey adjustment for multiple comparison. *P*-values < 0.05 were considered statistically significant. Unsupervised hierarchical clustering analysis of the log 2-transformed proteomic data was carried out using the Ward method in JMP v5.1 (SAS Institute). GraphPad Prism 6 (San Diego, CA) was used to fit curves to concentration-dependent and time-dependent cell viability (growth) data, and the IC_{50} values were determined from these generated curves.

RESULTS

Fluoxetine modulates the RTK/Akt/mTOR and RTK/ERK pathways

While the anti-proliferative and apoptotic effects of FLX in various malignant cell lines have been demonstrated, there appears to be no common signaling pathway(s) modulated by this drug. Given the heterogeneity of breast cancer, we hypothesized that the mechanism of action of FLX would be different for each subtype of breast cell line. Here, we examined the expression levels of several proteins encompassing the broad growth factor-mediated signaling and apoptotic pathways by high-throughput RPPM. We performed RPPM on basal normal (HMEC-15 and late passage MCF10A), triple-negative (SUM149PT and DKAT), luminal A (T47D), luminal B (BT-474 and R-BT474), and HER2+ (Au565) cell lines. The unsupervised hierarchical clustering analysis segregated the samples into distinct clusters of luminal (R-BT474, BT474, Au565, T47D) and basal-like cell lines (HMEC, MCF10A, SUM149PT, and DKAT) (Figure S1), which are consistent with the gene expression analysis of breast cancer cell lines[29]. There was also a distinct clustering of the HER2+ cell lines (R-BT474, BT474, and Au565) as previously described[30]. In contrast, there was no clear partitioning of samples into treatment groups (*i.e.*, control *vs* FLX).

Next, we determined the effects of 10 μmol/L FLX treatment on protein expression changes across cell lines by one-way ANOVA. Here, we limit our statistical analysis to triple-negative SUM149PT, luminal T47D, HER2+ Au565, and normal late passage (Late) MCF10A. Given the mixed population of HMEC-15 (*i.e.*, cobblestone *vs* large, flattened morphology) that

we encountered during RPPM lysate preparation, Late MCF10A data were used in our analysis. As Figure 1A indicates, the FLX-induced changes in protein levels of Akt S473, p70 S6K T389, and eIF4G S1108 were significantly different across cell lines after 24 h. These proteins are components of the RTK/Akt/mTOR pathway, which plays a vital role in cell proliferation, growth, and survival.

The Akt S473 levels in SUM149PT consistently decreased after 24 h and 48 h of FLX treatment, while the expression in Late MCF10A cells increased (Figure 1). For both T47D and Au565 cells, treatment resulted in no change in baseline Akt S473 at 24 h, but a small decrease in protein levels after 48 h. Interestingly, SUM149PT showed an increase in activated glycogen synthase kinase 3 (GSK3)α/β Y279/Y216 after 48 h of treatment. This effect could be explained by a decrease in activity of Akt, which would otherwise inactivate GSK3. In contrast, the increased GSK3α/β Y279/Y216 levels in Late MCF10A may be attributed to some other mechanism, which remains to be determined.

Downstream of Akt are important regulators of protein synthesis, namely the p70 S6K and eIF4G. In all cell lines, FLX induced a decrease in both proteins at 24 h, suggesting inhibition of translation (Figure 1A). Although not statistically significant, the activation of master regulator of cell growth, mTOR S2448, was inhibited by FLX in all cell lines, which is consistent with decreased p70 S6K activity. The translational inhibition was sustained up to 48 h as evidenced by a decrease in eIF4G S1108 (Figure 1B) as well as p70 S6K T389 by Western blot (Figure 1C).

To date, only few studies have shown FLX-mediated inhibition of ERK, and this effect appears to be cell type-dependent[20,31]. Here, we showed that after 48 h, FLX had different effects on ERK1/2 T202/Y204 level of each breast cell line (Figure 1B). SUM149PT and T47D showed inhibition of ERK1/2 after 48 h of treatment, while Late MCF10A and Au565 showed ERK1/2 activation, which we also confirmed by Western blot (Figure 1C).

Fluoxetine modulates mammary epithelial cell growth

Several groups have shown that FLX treatment can lead to growth inhibition or death of cancer cells, although not much is known about its effect on breast cancer subtypes. We assessed the effect of FLX on cell viability or growth of the aforementioned cell lines, using various concentrations at different times. Although MCF10A originated from the same mastectomy fibrocystic diseased tissue, several variation of this cell line exist[32]. Initially, we obtained the Late MCF10A cells, which are spindle in shape (Figure 2A). A lower passaged MCF10A cells (Early MCF10A) were also obtained, and the derived epithelial cells have cobblestone morphology. In comparison to untreated (control) cells, FLX treatment reduces cell growth in a time-dependent and dose-dependent manner, with the biggest changes in IC_{50} occurring between 24 h and 48 h for most cell lines (Figure 2B-F). At 48 h, IC_{50} ranges from 6.8-10.7 μmol/L across cell lines. In our subsequent analysis and experiments, we used a fixed dose of 10 μmol/L to assess the mechanism of action of FLX.

In comparison to control cells, the FLX-treated SUM149PT and Late MCF10A showed a decreased ability to reduce MTT to formazan crystals, suggesting significant cell growth inhibition by 48 h (Figure 3, red and blue solid lines *vs* red and blue dotted lines). In contrast, the treated Early MCF10A continued to grow albeit at a slower rate than control cells (green dotted *vs* solid lines) even after 48 h of treatment. The FLX-treated T47D and Au565 cells also showed cell growth reduction, which was greater than treated Early MCF10A (Figure 3, compare black and purple dotted lines *vs* green dotted lines). The effects of FLX on T47D and Au565 over time suggest that the drug is acting as a cytostatic rather than cytotoxic agent.

Fluoxetine induces autophagy and ER stress in rapidly dividing cells

In this study, we showed that both rapidly dividing SUM149PT and Late MCF10A cells are most sensitive to FLX-induced cell growth inhibition (Figure 3). The decreased protein synthesis in both cell lines at 24 h of FLX treatment (Figure 1, p70 S6K and eIF4G) suggests altered energy metabolism, which can contribute to cell growth inhibition and even cell death.

Our RPPM data suggested that mTOR activity was inhibited by FLX by 24 h (Figure 1A). Inhibition of mTOR is mediated by adenosine monophosphate kinase (AMPK) during low cellular energy status or stress, which is then followed by autophagy[2,9,33]. We confirmed by Western blot that the central metabolic sensor AMPK was activated in FLX-treated SUM149PT and Late MCF10A as early as 2 h and up to 24 h, suggesting metabolic stress in these cells (Figure 4A). Next, we examined the expression of cleaved microtubule-associated protein 1 light chain 3 (LC3-Ⅱ), which is required for autophagosome transport and maturation as well as a well-accepted monitor of autophagy[2,9]. After 24 h of FLX treatment, only SUM149PT showed increased level of LC3-Ⅱ (data not shown). By 48 h, the LC3-Ⅱ level in SUM149PT was significantly elevated compared to Late MCF10A (Figure 4A). Meanwhile, autophagy was not induced in Early MFCF10A.

Induced cellular stress results in orchestration of several processes that dictate whether cells live or die. These processes include autophagy, ER stress, and apoptosis. The link between these processes has been elucidated only in the past 9 years. An important regulator and sensor of ER stress is BiP, which maintains proper protein folding and helps restore misfolded proteins[34]. In our study, only SUM149PT showed an apparent increase in BiP following exposure to FLX (Figure 4B). But UPR was induced in both FLX-treated SUM149PT and Late MCF10A, as indicated by an increase in PERK-mediated activation of translation

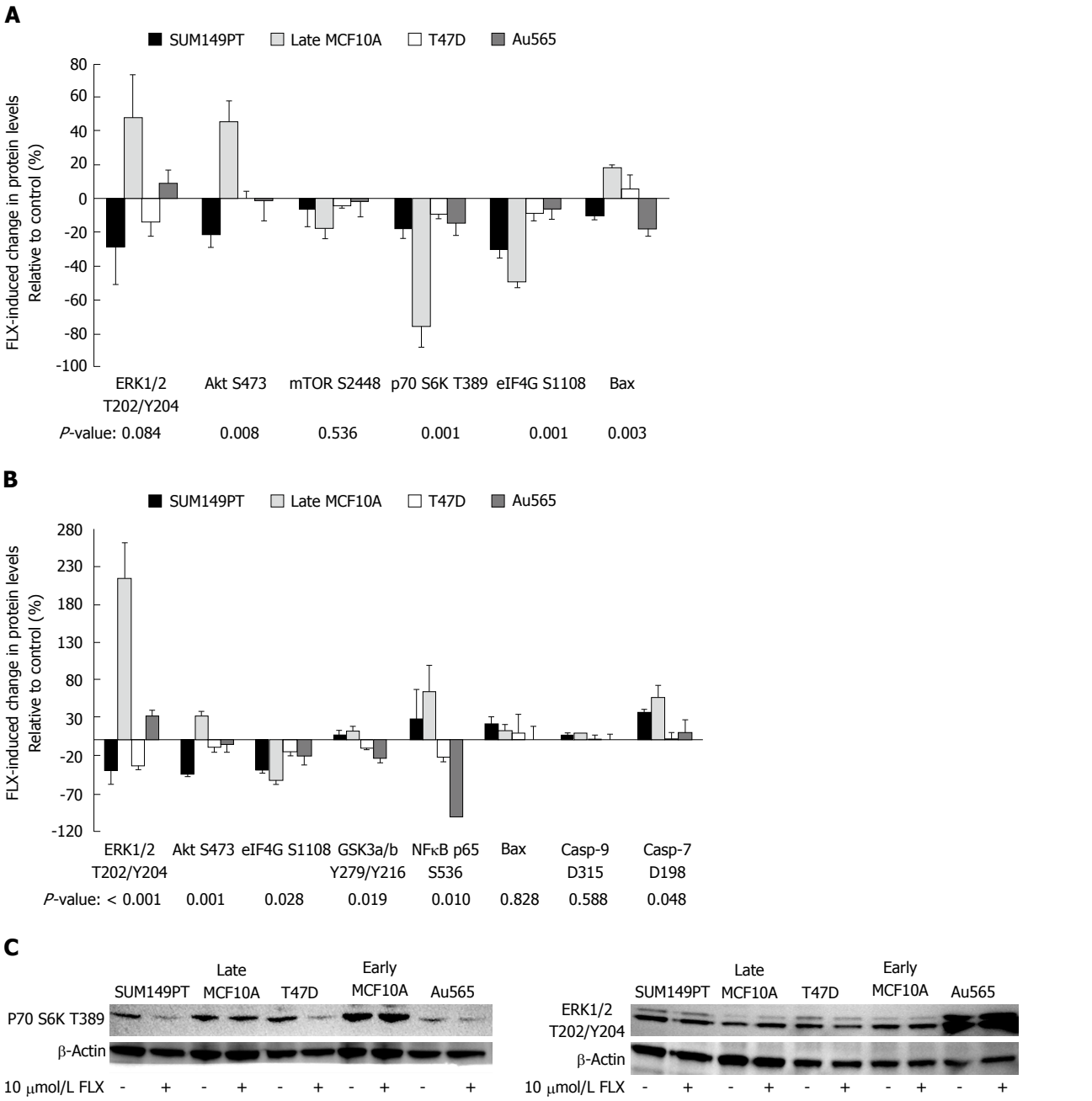

Figure 1 Expression levels of proteins along the phosphoinositide 3-kinase/Akt/mammalian target of rapamcyin, mitogen-activated protein kinase/extracellular signal-regulated kinase, and apoptosis pathways vary by cell lines. Statistically significant and differentially expressed proteins, following (A) 24 h and (B) 48 h treatment of 10 μmol/L FLX, were indicated with stars; (C) Expression of few selected proteins across cell lines after 48 h of 10 μmol/L fluoxetine was confirmed by Western blotting. For detection of p70 S6K and ERK1/2 activation, cell extracts were loaded at 100 μg and 30 μg per lane, respectively. MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; FLX: Fluoxetine; p70 S6K: p70 S6 Kinase; GSK3: Glycogen synthase kinase 3; eIF4G: Eukaryotic translation initiation factor 4G.

initiation factor eIF2α at S51. Activation of eIF2α has been shown to increase NFκB activity as well as inhibit protein synthesis[35,36]. Both RPPM and Western blot data indicated an increase in NFκB activity in SUM149PT and Late MCF10A after 48 h of FLX treatment (Figure 1B and 4B). The increased eIF2α S51 levels in both cell lines were also consistent with the inhibition of eIF4G-mediated protein synthesis (Figure 1B). Meanwhile, the Early MCF10A did not show further increase in BiP, PERK, eIF2α S51, and NFκB p65 S536 (Figure 4C), suggesting no appreciable UPR in these normal cells with FLX treatment.

Although previous study has linked the activation of NFκB to autophagy through modulation of essential autophagy gene *Beclin-1*[37], our study did not show a change in basal Beclin-1 protein levels with FLX treatment (data not shown). This suggests that Beclin-1 expression in mammary epithelial cells is not dependent on NFκB activation. However, a plausible link between UPR and autophagy induction in FLX-treated SUM149PT

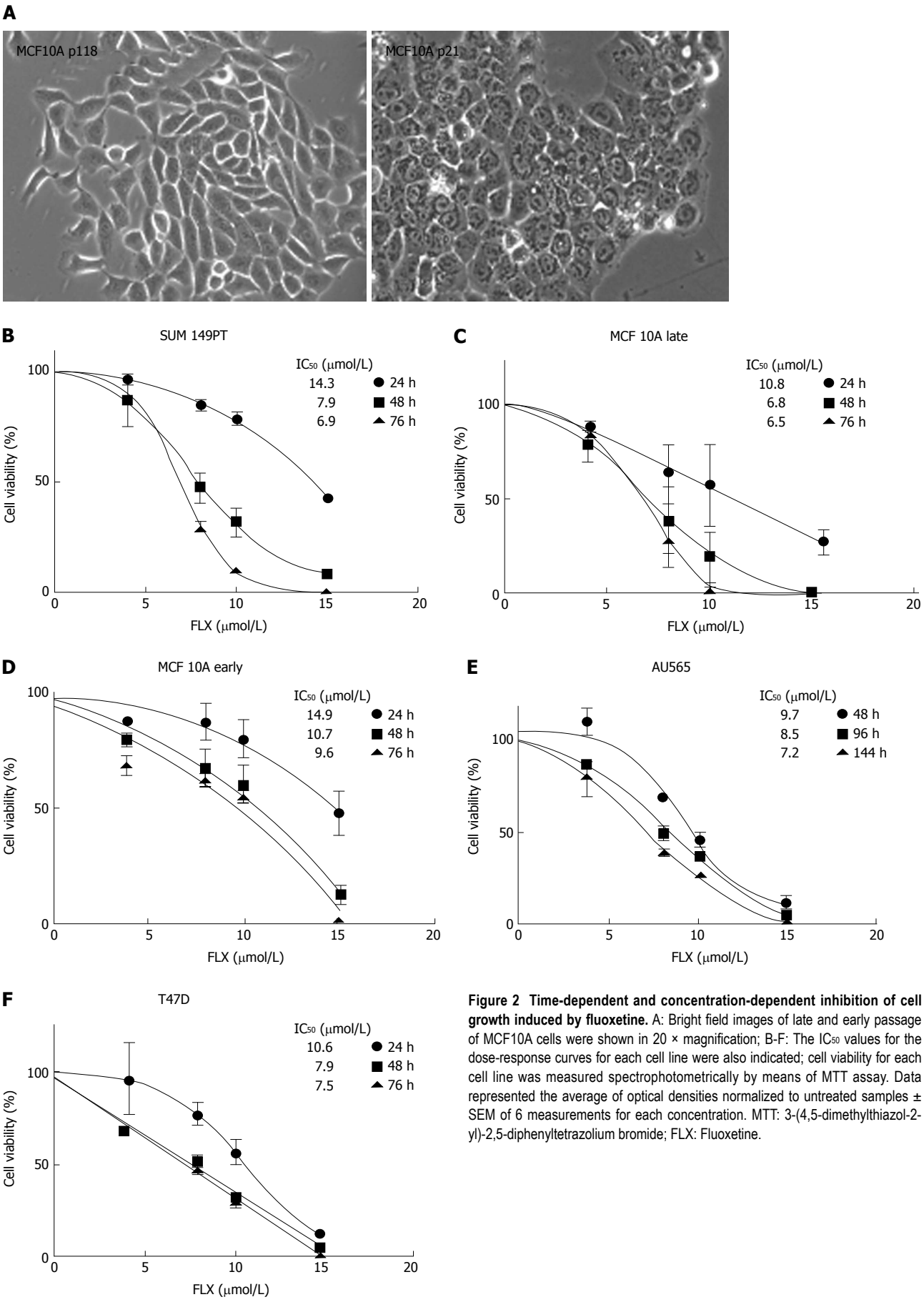

Figure 2 Time-dependent and concentration-dependent inhibition of cell growth induced by fluoxetine. A: Bright field images of late and early passage of MCF10A cells were shown in 20 × magnification; B-F: The IC_{50} values for the dose-response curves for each cell line were also indicated; cell viability for each cell line was measured spectrophotometrically by means of MTT assay. Data represented the average of optical densities normalized to untreated samples ± SEM of 6 measurements for each concentration. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FLX: Fluoxetine.

is BiP, which has been shown to be necessary in the maturation step of autophagic vesicle (Figure

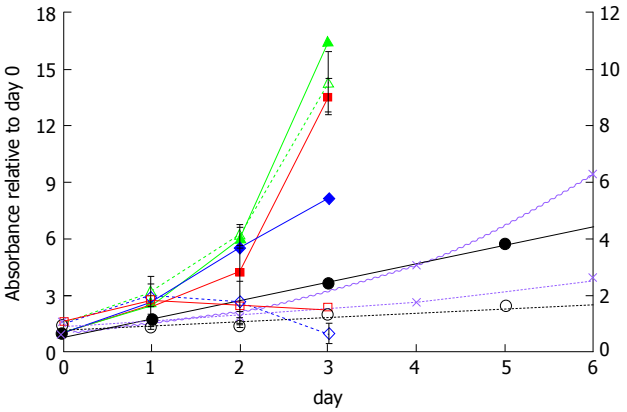

Figure 3 Effect of incubation time on microwave theory and techniques reduction by cell lines in the absence (solid lines) and presence (dotted lines) of 10 μmol/L fluoxetine. Reduction of MTT by viable Late MCF10A (diamonds), SUM149PT (squares), Early MCF10A (triangles), T47D (circles), and Au565 cells (crosses) was monitored at absorbance of 570 nm and normalized to day 0. The ordinate axis on the right represents the absorbance values for T47D and Au565 cells. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

5), downstream of Beclin-mediated membrane nucleation[9,10].

Cells undergoing prolonged autophagy and ER stress eventually succumb to death. Our RPPM data indicated an increase in pro-apoptotic Bax in Late MCF10A after 24 h of FLX (Figure 1A) as well as activated caspase-7 at 48 h in both SUM149PT and Late MCF10A (Figure 1B), suggesting that both cell lines undergo apoptosis. We confirmed caspase-7 activity by monitoring PARP cleavage, which was significant only in SUM149PT after 48 h of FLX treatment (Figure 4C). The lack of cleaved PARP in treated Late MCF10A does not necessarily correlate with inactive caspase-7. Rather, PARP cleavage site is either modified in this particular cell line or inaccessible by the antibody used in the Western blot analysis. Meanwhile, FLX did not induce apoptosis in Early MCF10A.

During ER stress, calcium released into the cytoplasm may enter the mitochondria to induce the intrinsic pathway to apoptosis[38]. Few members of the caspase family have been implicated in ER stress conditions. ER-resident procaspase-12 is cleaved or activated by protease calpain in response to calcium release[39]. Another study suggested that translocation of caspase-7 from the cytoplasm to the ER can cleave caspase-12 and mediate cell death[40]. In our study, there were no significant changes in calpain and cleaved caspase-12 levels in any of the cell lines with FLX treatment (Figure 4C). This data suggests that the observed FLX-induced cell death in SUM149PT and Late MCF10A is mediated through the mitochondrial apoptotic pathway.

Persistent UPR and autophagy in SUM149PT promotes apoptosis

To closely determine the effects of FLX treatment in the aggressive TNBC line SUM149PT, the protein levels of the aforementioned regulators of ER stress, UPR, autophagy, and apoptosis were monitored at different times by Western blots. As Figure 4D indicated, the metabolic sensor AMPK was activated as early as 2 h of FLX treatment. Modest but increased expression levels of BiP, eIF2α S51, and LC3-Ⅱ at 24 h indicated concurrent induction of UPR and autophagy, which were sustained up to 48 h. As a consequence of excessive ER stress, cell death mediated by mitochondrial apoptosis ensues. The increase in caspase-7 activation, as monitored by PARP cleavage (Figure 4C), coincided with a decrease in anti-apoptotic Bcl-2 level (Figure 4D). In FLX-treated SUM149PT, we would expect that the negative regulation of Beclin by Bcl-2 at the ER surface would be negligible and further support autophagy induction, given the role of Beclin in membrane nucleation[38].

Although FLX-treated SUM149PT showed UPR at 48 h (Figure 4D), we could not detect levels of CHOP and GADD34 that are known to promote apoptosis as a result of prolonged ER stress (data not shown). The absence of these proteins suggests instability, as previously shown in mouse embryonic fibroblasts (MEFs) that have been treated with classical inducers of ER stress, such as thapsigargin (TG) and tunicamycin (TM)[41]. In this study, Rutkowski *et al*[41] demonstrated that CHOP was rapidly degraded with a half-life of 4 h or less, while BiP expression was robust with a half-life of about 48 h. Lack of CHOP expression in the time points tested in our study was not surprising. Given the high dose of FLX (10 μmol/L) used in SUM149PT compared to the low dose of TG (2.5 nmol/L) or TM (30 nmol/L) in MEFs suggests that FLX is a less potent inducer of UPR than either TG or TM. Comparison of CHOP and GADD34 protein levels in an *in vivo* model treated with either classical UPR inducers or FLX will be an important future direction of this study.

Fluoxetine affects cell cycle progression

Cells that undergo UPR are subjected to global translation repression and cell cycle arrest[42]. To determine which phase of the cell cycle is modulated by 10 μmol/L FLX treatment, we performed FACS analysis on cells that undergo significant UPR and apoptosis. T47D cells were used for comparison, given the cytostatic (*vs* cytotoxic) effect of FLX in this cell line (Figure 3). As Table 1 indicates, the proportion of SUM149PT and Late MCF10A cells entering the DNA synthesis (S) and mitosis (G2/M) phase decreased significantly with time, along with an increase in the growth (G1) phase. Meanwhile, FLX treatment in T47D did not change any phase of the cell cycle. Together, these results suggest that FLX-induced UPR in SUM149PT and Late MCF10A is associated with G1 arrest, which is consistent with previous studies that described the effect of FLX in colon (HT29), breast (MDA-MB-231), and cervical (SiHa) cancer cell lines[20,22].

DISCUSSION

In the present study, we showed that the treatment of TNBC line SUM149PT with antidepressant fluoxetine induces autophagy (Figure 4A) with concomitant decrease in cell growth (Figures 2 and 3). The activation

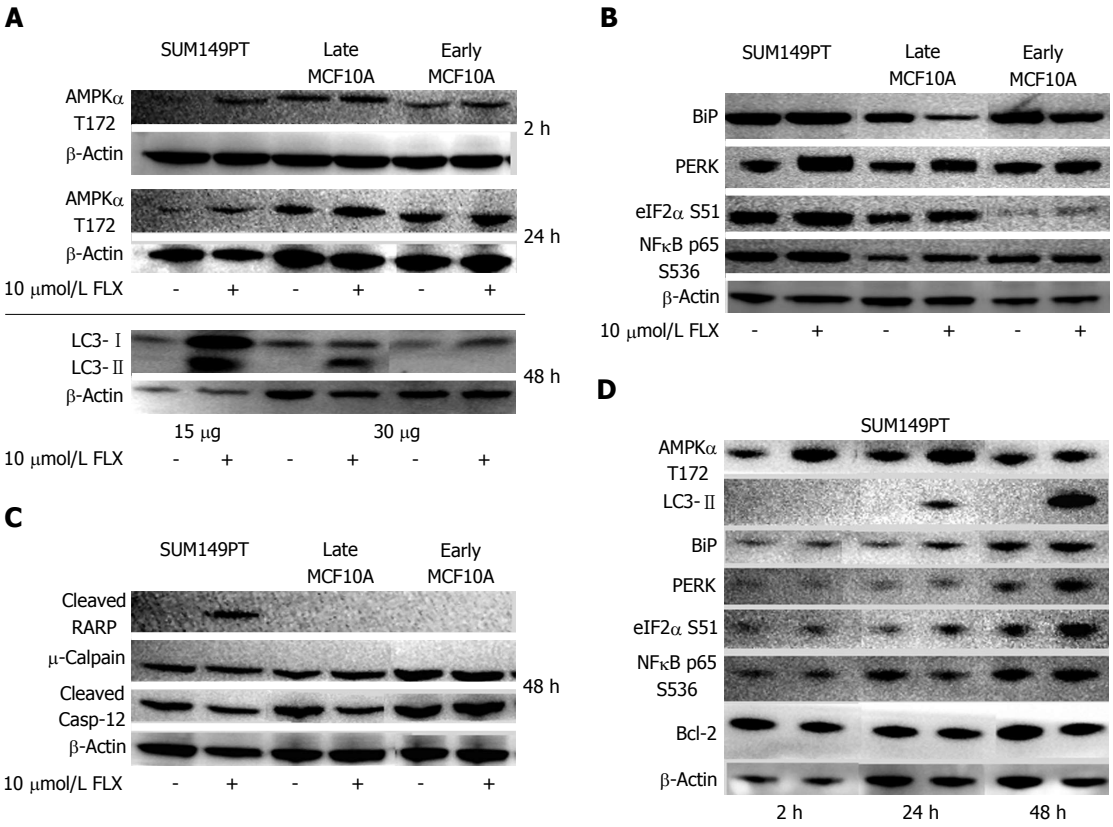

Figure 4 Key proteins along the autophagy, unfolded protein response, and apoptosis pathways were examined across cell lines after 10 μmol/L fluoxetine treatment at various times. A, D: Critical effector of autophagy, AMPK, was activated after 2 h and 24 h of treatment in SUM149PT. Autophagy in cell lines was detected by the presence of cleaved LC3 (LC3- Ⅱ) bands; B, C: The balance, level, and duration of ER stress sensors (B) and effectors of UPR (C) may ultimately dictate whether a cell lives or dies; D: Protein levels were monitored for up to 48 h in SUM149PT after fluoxetine treatment, showing concurrent induction of UPR and autophagy by 24 h. Blots shown were representative of at least 3 different experiments. The amounts of total cell extracts loaded for each cell line were indicated in the bottom panels in (A) for cleaved LC3 detection, while total loading in all cell lines for AMPKα detection was 100 μg. In panels B, C, and D, the amount of cell extracts loaded in each lane was 30 μg, 100 μg and 50 μg, respectively. Since the Abcam antibody for cleaved LC3 detection was very sensitive in panel A, we chose another manufacturer (cell signaling technology) to detect the same cleaved protein in panel D without the problem of overexposure during chemiluminescence. UPR: Unfolded protein response; ER: Endoplasmic reticulum; AMPK: Adenosine monophosphate kinase.

of AMPK, but a decrease in Akt and ERK activation, are likely to contribute to FLX-mediated cytotoxic autophagy in this cell line as early as 24 h (Figures 1, 2, and 4A). In contrast, the autophagy in Late MCF10A after 48 h of treatment may be dependent on ERK activation as previously reported for breast cell lines[43,44]. However, unlike those cells with survival advantage, Late MCF10A growth was significantly inhibited. Whether or not this growth inhibition is due to nuclear translocation of ERK to promote p53 upregulation and subsequent apoptosis, as has been suggested in some models[45], remains to be determined.

Both SUM149PT and Late MCF10A are rapidly dividing cells (Figure 3) and have increased metabolic demands. The FLX-induced decrease in protein synthesis mediated by p70 S6K or eIF4G is likely to contribute to metabolic stress, thereby promoting autophagy in these cells. Given that the spindle-shaped Late MCF10A used in our study may have undergone some biochemical and/or genetic changes due to continual passaging, we acquired a different MCF10A line that has not been passaged extensively and shows normal cobblestone morphology (*i.e.*, Early MCF10A). The 48 h FLX treatment did not induce autophagy (Figure 4A) but reduced cell growth in Early MCF10A (Figure 2) at a smaller proportion (40.1%) compared to Late MCF10A (81.3%) and SUM149PT (68.2%).This result is consistent with chemical-induced autophagy that is selective for only transformed mammary epithelial cells[44]. In preliminary testing of another TNBC line, MDA-MB-231, we found that the FLX-induced cytotoxicity in these cells was also associated with autophagy induction (data not shown). To our knowledge, this study is the first report of FLX-induced autophagy that results in significant growth inhibition of aggressive TNBC line.

In addition, the observed FLX-induced autophagy may be the result of ER stress and subsequent induction of UPR, consisting of PERK-dependent phosphorylation of eIF2α (Figure 4B), which can lead to translation inhibition of IκBα and subsequently NFκB activation[35,36]. Our RPPM data indicated residual IKK activity, as measured by IκBα S32/S36, even after 48 h of FLX treatment (data not shown), which promotes proteasomal-mediated degradation of IκBα, followed by NFκB translocation to the nucleus and subsequent activation. The FLX-induced NFκB activation in SUM149PT (Figure 1B),

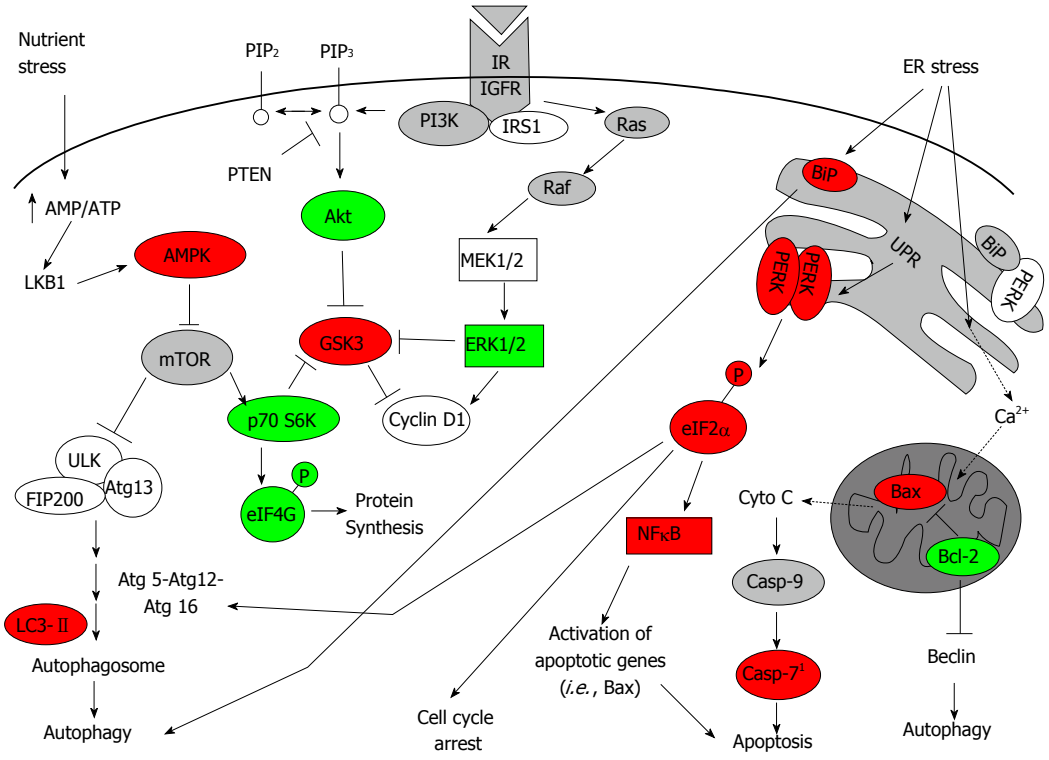

Figure 5 Integrated signaling network modulated by fluoxetine in inflammatory triple negative breast cancer line SUM149PT. Proteins with increased and decreased expression levels after FLX treatment were highlighted in red and green, respectively. This proposed that model of FLX-induced cellular stress results in excessive UPR that leads to cell growth inhibition, autophagy, and potentially selective promotion of NFκB-mediated transcription of apoptotic genes, such as Bax. Oligomerization of Bax promotes the release of cytochrome C into cytosol and subsequent activation of caspase-9 and downstream effectors of apoptosis, such as caspase-7[38,40]. Alternatively, Ca^{2+} released during ER stress has been shown to promote cytochrome C release. AMPK: Adenosine monophosphate kinase; UPR: Unfolded protein response; FLX: Fluoxetine; p70 S6K: p70 S6 Kinase; ER: Endoplasmic reticulum.

as determined by post hoc analysis of ANOVA, was significant compared to slowly dividing T47D and Au565 cells ($P < 0.05$), but comparable to Late MCF10A ($P > 0.05$). The elevated NFκB activation during ER stress is expected to increase selective transcription of stress response genes[8]. The identities of those genes in our preclinical study have yet to be determined.

The cytotoxic effect of FLX in SUM149PT and Late MCF10A is associated with cell cycle arrest at the G1 phase as early as 24 h (Table 1). This is consistent with the inhibition of global protein translation as a result of ER stress. In an attempt to mitigate such stress, autophagy is induced in rapidly dividing SUM149PT and Late MCF10A. When each cell's basal metabolic machinery could no longer be maintained for survival, cell death ensues. In our study, prolonged stress and sustained autophagy leads to activation of caspase-7 (Figures 1B and 4C), which has been shown in previous studies to be an effector of ER stress-induced cell death[40]. As suggested by others[39,40,46], we cannot rule out that the Ca^{2+} release from the ER during UPR may promote apoptosis, perhaps through activation of a different calpain isoform and subsequent proteolytic processing of a caspase other than caspase-12. Moreover, ER-localized Bcl-2 has been shown to regulate the amount of Ca^{2+} released from the ER to cytosol during stress[38]. In SUM149PT, the decreased Bcl-2 after 48 h of FLX treatment further supports the activation of apoptosis (Figure 4). Taken together, our study suggests that the FLX-induced UPR and autophagy in rapidly dividing SUM149PT and Late MCF10A promotes apoptosis, thus showing the intricate crosstalk between ER and mitochondria. Despite the network complexity, observations reported by us and others provide further evidence how individual components of UPR, autophagy, and apoptosis coordinately regulate cell fate (Figure 5). In regards to FLX-induced UPR in SUM149PT after 48 h, we hypothesized that activation of NFκB may promote transcription of apoptotic genes (*i.e.*, Bax) over cell survival genes (*i.e.*, Bcl-2), thereby favoring cell death through the mitochondrial pathway.

There are several limitations in the present study that precluded us from making general conclusions about the effects of FLX in molecular subtypes of breast cancer. First, each cell type has innate adaptive response to ER and metabolic stress. The extent to which cells will either adapt to stress or die will depend on (1) the genetic/biochemical makeup that dictates proper cellular response, (2) time of exposure to external and/or internal stress stimuli, and (3) the delicate balance between cell survival or death promoting genes. Second, the extent of cytotoxic response in basal-like SUM149PT may not be similar to other subtypes of TNBC that have been recently described[47]. Third, comparison of cytotoxic profiles between FLX and classical inducers of ER stress was

Table 1 Proportion of cells in each cycle phase in the absence and presence of fluoxetine

	SUM149PT Control	SUM149PT 10 μmol/L FLX	Late MCF10A Control	Late MCF10A 10 μmol/L FLX	T47D Control	T47D 10 μmol/L FLX
24 h						
Sub G	1.1	1.72	0.66	1.77[2]	1.86	1.63
	(0.34)[1]	(0.55)	(0.13)	(0.2)	(0.26)	(0.18)
G1	39.6	62.41[2]	60.26	79.89[2]	56.03	59.21
	(0.74)	(1.02)	(1.59)	(0.71)	(2.05)	(0.55)
S	34.64	20.18[2]	23.78	10.75[2]	21.14	18.26
	(1.28)	(1.11)	(1.16)	(0.43)	(1.12)	(1.45)
G2/M	25.26	16.12[2]	15.8	7.86[2]	21.44	21.39
	(0.79)	(0.3)	(0.65)	(0.13)	(0.98)	(1.32)
48 h						
Sub G	1.91	6.67	0.41	10.26[3]	1.7	2.66
	(0.42)	(2.47)	(0.05)	(0.52)	(0.32)	(0.39)
G1	46.79	67.73[3]	65.48	79.77[3]	57.52	62.35
	(1.94)	(1.16)	(0.93)	(1.3)	(2.01)	(1.31)
S	29.69	14.01[2]	22.24	6.59[3]	22.33	18.07
	(2.79)	(0.84)	(0.6)	(0.72)	(1.54)	(1.19)
G2/M	22.24	11.90[2]	12.31	3.58[3]	18.95	17.32
	(1.08)	(2.64)	(0.43)	(0.13)	(0.24)	(0.29)

[1]Numbers in parentheses represent standard errors; [2]*P*-value < 0.05 compared to matching control; [3]*P*-value < 0.001 compared to matching control; FLX: Fluoxetine.

not performed in the present study. Xenograft models of SUM149PT and another TNBC subtype will be an important follow-up *in vivo* study to obtain important insights to the mechanism of action of FLX and potent ER stress inducer, thapsigargin.

In summary, our study demonstrated the complex actions of FDA-approved drug in malignant mammary epithelial TNBC cells that go beyond the inhibition of selective serotonin re-uptake. In addition to its utility for treating clinical depression, FLX has been used to improve quality of life in cancer patients[48]. Recently, FLX has been shown to reverse the multidrug resistance in cancer cells, enhancing the apoptotic effects of chemotherapeutics[23]. Here, we employed high-throughput RPPM to monitor FLX-induced changes in the expression of proteins encompassing the RTK/Akt/mTOR, MEK/ERK, and apoptotic pathways to complement our functional data. Our data analysis pointed to few proteins that play a role in cellular homeostasis and stress response. These proteins are components of the highly integrated autophagy, UPR, and apoptosis in response to ER and metabolic stress. The apparent sensitivity of TNBC SUM149PT to stress-mediated apoptosis has important clinical implications, given the aggressive biology of the inflammatory breast tumor that the cell line was originally derived and frequency of therapeutic resistance. Currently, a multi-modal approach (systemic chemotherapy, surgery, and radiation) is used to treat inflammatory breast cancer. Given the safety profile[49], potential as a chemosensitizer[23], and induction of ER stress-mediated apoptosis (Figure 5), FLX may provide additional benefit to current treatment modality for inflammatory TNBC. The anti-proliferative effect of FLX alone and in combination with chemotherapeutic will have to be tested in an *in vivo* model of TNBC in the near future.

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COMMENTS

Background

The ability of widely prescribed antidepressant fluoxetine to induce cell death or chemosensitize cancer cells has been described previously, but the mechanism of action is not well understood and appears to be cell type-dependent. While the inhibition of extracellular signal regulated kinase pathway and cell cycle progression has been proposed in two different breast cancer cell lines, the comparative studies did not employ non-transformed breast epithelial cells as additional control. Thus, information regarding the selectivity of fluoxetine-induced growth inhibition in molecular subtypes of breast cancer and normal breast cells is lacking.

Research frontiers

Given the heterogeneity of breast cancers, including the aggressive triple negative breast cancer subtypes, efforts to identify targets of therapeutic intervention are greatly needed to improve clinical outcome and survival of women diagnosed with such phenotype.

Innovations and breakthroughs

The authors' study is the first to describe the selective cytotoxicity of fluoxetine for basal-like inflammatory triple negative breast cancer (TNBC) cells over non-transformed mammary epithelial cells that involves the unfolded protein response and autophagy pathways.

Applications

Inflammatory breast cancers have a high likelihood of residual disease and recurrence. The ability of fluoxetine to promote unfolded protein response, autophagy, and subsequent death in our preclinical model of inflammatory breast cancer may not only alleviate psychological stress but also potentially reverse therapeutic resistance. The utility of FLX as a potential adjuvant to treatment regimen of inflammatory breast cancer will have to be evaluated in zenograft models of TNBC.

Terminology

Unfolded protein response (UPR) and autophagy are both physiological responses to oxidative and metabolic stress with the goal of restoring balance in correctly folded proteins and energy sources, respectively. However, prolonged cellular stress can lead to apoptosis. Because UPR and autophagy promote survival and death outcomes, mechanistic insights to their inter-dependent functions may lead to development of new treatment strategies against many diseases where both processes have been implicated.

Peer-review

The paper is good, it has accomplished with many different cell lines.

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