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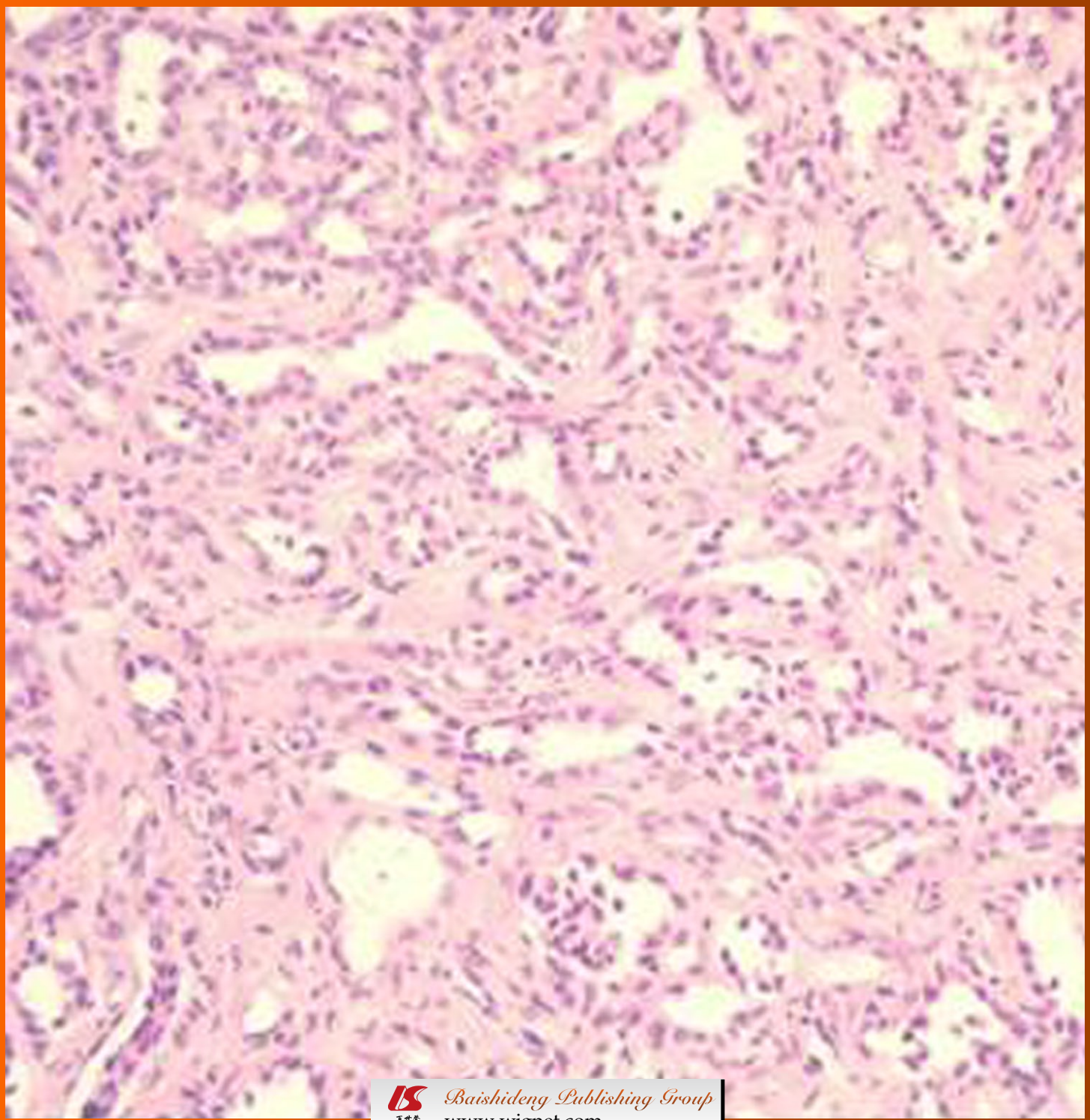

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REVIEW

Analysis of the Hox epigenetic code

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Abstract

Archetypes of histone modifications associated with diverse chromosomal states that regulate access to DNA are leading the hypothesis of the histone code (or epigenetic code). However, it is still not evident how these post-translational modifications of histone tails lead to changes in chromatin structure. Histone modifications are able to activate and/or inactivate several genes and can be transmitted to next generation cells due to an epigenetic memory. The challenging issue is to identify or "decrypt" the code used to transmit these modifications to descent cells. Here, an attempt is made to describe how histone modifications operate as part of histone code that stipulates patterns of gene expression. This papers emphasizes particularly on the correlation between histone modifications and patterns of *Hox* gene expression in *Caenorhabditis elegans*. This work serves as an example to illustrate the power of the epigenetic machinery and its use in drug design and discovery.

Key words: Epigenetic code; Histone code; Histone modifications; *Hox* gene expression

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INTRODUCTION

The basic unit of chromatin corresponds to DNA that is packaged into periodic nucleoprotein structures known as nucleosomes[1]. The nucleosome comprises an octamer of eight core histone proteins (two H2A, H2B, H3 and H4) around which 146 base pairs of dsDNA are wrapped in 1.65 left-handed superhelical turns[2]. Histone H1 serves as a linker protein and directs the formation of a higher-order structure in the nucleosomal array. The histone N-terminal tails comprise between 25 and 40 residues and are exposed on the surface of the nucleosome. The amino acid sequences of these N-terminal tails are highly conserved, possibly due to the roles played by a number of important post-translational modifications at these sequences[3].

A number of selected amino acid residues are subject to a variety of enzyme-catalyzed posttranslational modifications. These modifications include acetylation and methylation of lysines (K) and arginines (R), phosphorylation of serines (S) and threonines (T)[4,5], which are carried out by a variety of chromatin modifying complexes, such as the COMPASS (for histone methylation), NuA4/Tip60 (for Histone H4 acetylation), and NuA3 (for Histone H3 acetylation) complexes. All of these chromatin modifying complexes contain one of the histone modification enzymes, such as histone acetyltransferase (HAT), histone deacetylase (HDAC), histone methyltransferase (HMT), histone demethylase (HDMT), and histone kinase. These chromatin modification complexes work in concert with ATP-dependent chromatin-remodeling complexes, including the SWI/SNF, ISWI and NURD/

Mi-2/CHD complexes, which recognize specific histone modifications to restructure and mobilize nucleosomes.

Histone tails represent a complex set of epigenetic information. There are 50 distinct acetylated isoforms of the eight histone proteins[6]. In addition, several modifications can be applied to these isoforms including methylation of selected lysines and arginines (H3 and H4) and phosphorylation of serine (H3, H4, H2B). The methylation process includes the attachment of one, two, or three methyl groups. Other histone tail modifications also include ubiquitination and ADP-ribosylation[7]. The nucleosome surface is then decorated with thousands of these modifications, which could comprise a histone code[8,9] or an epigenetic code[10].

A challenging development occurs when a cell proliferates and generates two identical cells containing genes having the same status (expressed or repressed) as the ones in the mother cell. Every cell of an organism follows the same genetic code except germ cells and some cells of the immune system. Hence, the regulation of gene expression is not exclusively controlled by DNA but it is conducted in harmony with histones[11].

Cells control processes that permit them to remember the status of each gene before mitosis, and therefore preserve its phenotype. This transmission of gene expression patterns from mother cells to its descendants occur through a mechanism of gene bookmarking[12]. Hence, the cell state (identity) is kept safe within the structure of the chromatin and the epigenetic code.

Components of chromatin including DNA and histones undergo dynamic post-synthetic covalent modifications. The dynamic and not permanent post-translational modifications on histones represent epigenetic signatures and are created and removed whenever needed to alter the expression states of loci. These marks involve activities of modifying enzymes (writers), enzymes removing modifications (erasers), and readers of the epigenetic code. The erasers are crucial targets for manipulation to further understand the histone code and its role in biology and human disease[13,14].

The inheritance of epigenetic information is orchestrated by histone code readers (proteins that identify particular histone modifications) and histone code writers (proteins that duplicate the histone modifications).

Histone code readers and writers include structural domains such as the bromodomain, the chromodomain, and the plant homeodomain (PHD)[15]. These domains are required to recognize specific patterns of histone modifications including acetylation and methylation at given locations. The bromodomain is located mainly in HATs and chromatin remodeling proteins, whereas the chromodomain is found for example in HATs, HMTs, and HP1 (Figure 1). It has been shown that proteins involved in writing epigenetic information collaborate to maintain an epigenetic stability in the midst of great dynamic events at the molecular level[16].

Despite their phenotypic differences, *Caenorhabditis elegans* (*C. elegans*), a roundworm with a genome about 30

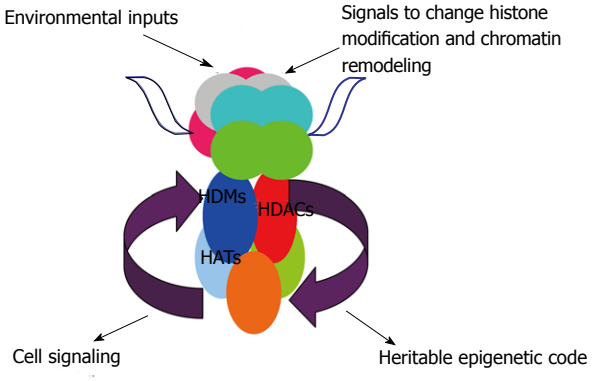

Figure 1 Histone modifications and chromatin remodeling. Environment factors and modifying enzymes are associated with regulating cell signaling and histone code. HDAC: Histone deacetylase; HAT: Histone acetyltransferase.

times smaller than human genome, however it encodes 22 000 proteins. In addition, approximately 35% of *C. elegans* genes are closely related to human genes, and both organisms have at least 80% amino acid sequence identity between their core histones[17]. For example, MES-2, the ortholog of human EZH2 has been reported to be a HMT for H3K27[18], and MES-4, a SET domain containing protein, has recently been shown to be required for H3K36 di-methylation (H3K36 me2) in mitotic and early meiotic germline nuclei and in early embryonic cells[19]. Whetstine *et al*[20] discovered the histone demethylase JMJD2A in mammalian cells and that has led to the identification of the *C. elegans* homolog, JMJD-2. This protein family was reported to be required in chemical methylation for H3K9/k36 me3.

There is accurate machinery that allows cells to recognize themselves and undertake specific tasks. This machinery represents the blueprint of various patterns of gene activation/inactivation throughout the cell cycle. Lack of expression or repression leads to an irregular outcome for the cell including altered genetic programs and increased rate of cell transformation[12]. This "know-how" is located mainly in the amino-terminal tails of the core histones[21]. The first association between a histone tail modification and a particular functional state of chromatin was reported by Pogo *et al*[22] and Hebbes *et al*[23]. It was shown that transcriptionally active chromatin fractions are enriched in acetylated histones, whereas regions of facultative heterochromatin and transcriptionally silent constitutive were located in underacetylated regions[24] .

As depicted in Figure 1, the set of histone tail modifications includes at least two subsets. The first subset represents the modifications that lead to on-going transcription and usually are classified as cell signaling, and the second subset represents the modifications that are heritable. This heritability of transcriptional states is the component that unambiguously identifies the histone code[19]. In addition, these histone modifications are also suggested to be used combinatorially to instruct genes for activation right after cellular differentiation[25,26]. This latter proposition could be used to model the pro-

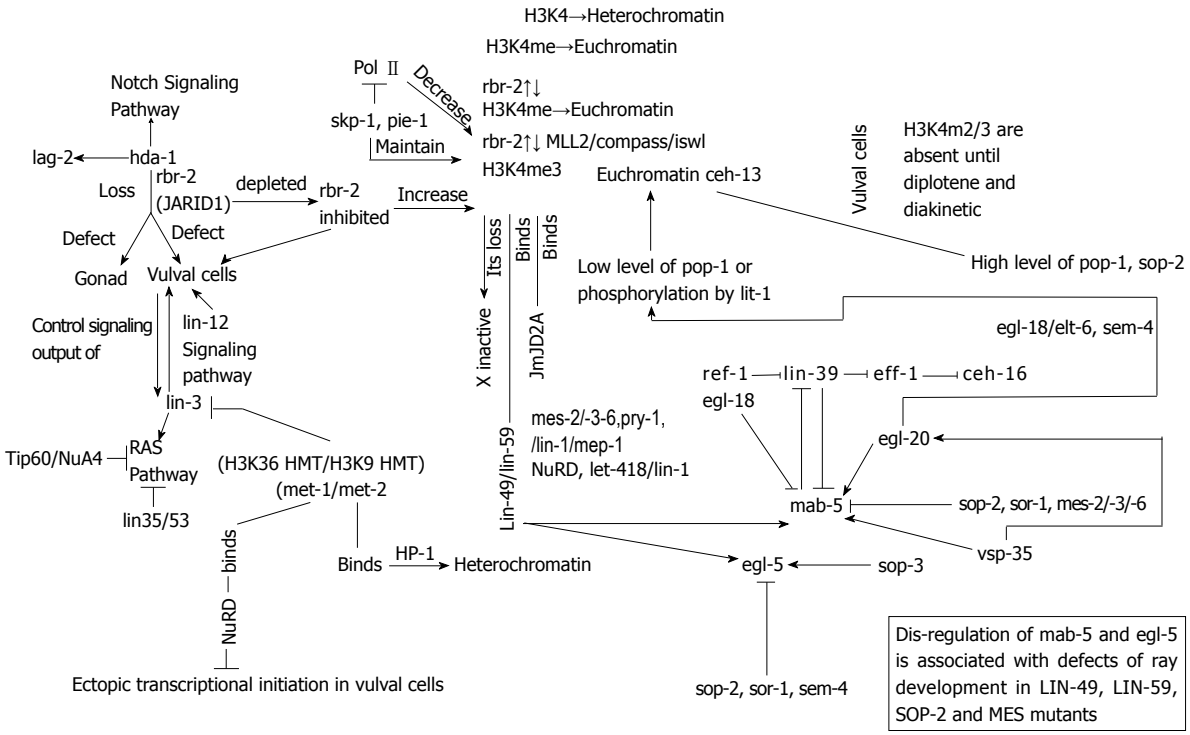

Figure 2 H3K4 modifications, Hox genes and cellular development.

Table 1 Methylation of H3K4, H3K9, H3K27 and H3K36

i	H3K4me(i)	H3K9me(i)	H3K27me(i)	H3K36me(i)
0	Off	On	On	Off
1	On	Off	Off	On
2	On	Off	Off	On
3	On	Off	Off	On

i=0 the me(i) part will be equal to zero which means that the histone is not methylated. Here the "On" state represents the transcriptionally euchromatin; and the "off" state represents the constitutive heterochromatin.

grammed activation of tissue-specific transcription factors throughout differentiation of ES cells[24].

Histone modifications associate closely with various biological functions. For example, as is depicted in Table 1, in *C. elegans*, methylation of H3K4/K36 correlates with transcriptionally competent euchromatin. Alternatively, methylation of H3K9/K27 correlates to a component of constitutive heterochromatin. Finding these patterns and the corresponding correlations with the transcriptional status of selected genes will lead the way to illustrate the process of an epigenetic code.

FROM *HOX* GENES TO HISTONE CODE

This study provides a detailed analysis of the Hox epigenetic code mainly in *C. elegans*. Methods used to propose such a code are based on a manual data mining approach and a thorough analysis of data gathered from various references and websites. A few discrepancies and contradictions were encountered during the design of Figures 2-4. For example, it is mentioned in a number of repositories for C. elegans that Sem-4 inhibits Lin-39. However, after investigating this issue further using the available literature and corresponding with many scientists, Sem-4 has been found to have an opposite role. Thus, patterns generated from data mining software should be manually checked to avoid similar discrepancies.

Although the histone code defined here targets a small organism, chromatin modifications in mammals including humans were used in this work to imply significant components of the Hox epigenetic code in *C. elegans*. In addition, there are many complexes that exist in both C. elegans and humans such as micro RNAs lin-4 and let-7 which have been connected to many cancers[27], and 153 kinase subfamilies which direct most cellular processes, particularly in signal transduction and coordination of complex pathways[28]. Similarities and homologs between both organisms are shown in Tables 2-5. Table 2 shows examples from the Ras-superfamily GTPases[29], Table 3 focuses on autophagy-related genes[30], Table 4 illustrates examples from the Ubiquitin-conjugating enzymes[31], and Table 5 shows specific similarities in *Hox* genes[32].

The development and maintenance of cellular identity is crucial in both embryonic and adult tissues for normal organ function. Hence the need to establish a stable transcriptional states within the cell, a process in which transcription factors have a vital role. One of those groups of transcription factors are known as *Hox* genes, representing a family of homeodomain-containing transcription factors that establish cellular identity during development, in addition to regulating numerous processes including apoptosis, receptor signalling, differentiation, motility and angiogenesis.

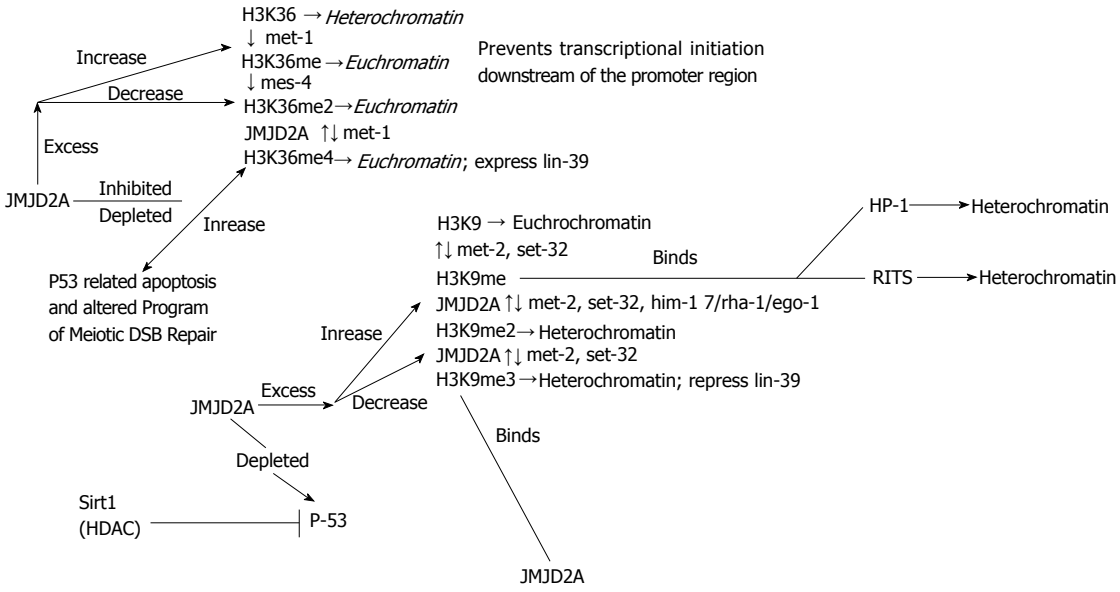

Figure 3 H3K36 modifications and cellular development. HDAC: Histone deacetylase.

Table 2 Known roles of Ras-superfamily GTPases in *Caenorhabditis elegans*: Homologs in humans

C.elegans gene name	Human homolog	Developmental role(s)
Ras/Rap/Ral family		
let-60	K-Ras	Cell fate determination, oocyte meiotic maturation, sex myoblast migration, axon pathfinding, olfaction, response to microbacterium nematophilum infection
Rho family		
rho-1	RhoA	Cytokinesis, hypodermal contraction, P cell migration, axon pathfinding
cdc-42	Cdc42	Cytokinesis, embryonic polarity, axon pathfinding
ced-10	Rac1	Gastrulation, cell migration (P cells, distal tip cells), cell corpse phagocytosis, neuronal migration, axon pathfinding, vulval morphogenesis
rac-2	Rac1	Neuronal migration, axon pathfinding
mig-2	Mtl	Cell migration (P-cells, distal tip cells, Q cells and descendants), neuronal migration, axon pathfinding, vulval morphogenesis
Rab family		
rab-1	Rab1	Innate immunity gene expression
rab-3	Rab3	Synaptic vesicle trafficking and release
Arf/Sar family		
arl-6	Arf6	Ciliary vesicle trafficking and Bardet-Beidl syndromes
evl-20	Arf2	Microtubule organization, vulval cell division, tissue morphogenesis
Ran family		
ran-1	Ran	Nuclear trafficking, nuclear reassembly, Kinetechore association with the mitotic spindle

Table 3 *Caenorhabditis elegans* orthologs of mammalian autophagy-related genes

C. elegans gene	Mammalian gene	Function
erp-1	*Bif-1*	Bax interacting factor that associates with Uvrag and Beclin 1
C33A11.4	*DRAM*	*p53*-induced damage-regulated
ced-9	*Bcl-2*	Anti-apoptotic protein, negative regulator of Beclin 1-mediated autophagy

Mammals have 39 *Hox* genes split between four groups of linked genes on different chromosomes and patterns of their deregulated expression have been reportered in cancer. This deregulation is tissue-specific, certain *Hox* genes that normally have tumour suppressive effects are silenced; and in other tissues, particular Hox genes are expressed in an abnormal temporospatial pattern with oncogenic effects[33]. Anomalies in *Hox* gene expression have been identified in abnormal development and malignancy, and re-expression in many cancers such as pancreatic cancer[34], leukemia[35], and neuroblastoma[36]. In some tumors, altered expression of Hox genes directly drives tumorigenesis through escape from apoptosis[37], alterations to receptor signalling[38], epithelial-mesenchymal transition (EMT)[39] and tumour cell invasion[40]. Therefore, Hox gene expression is a prospective diagnostic marker and therapeutic target.

Hox genes encode a family of transcription factors, are usually conserved within metazoans, and are involved in generating pattern along the anterior-posterior body axis. Their involvement occurs during early embryogenesis collinearly with their arrangement on the chromosome[41,42]. In *C. elegans*, the *Hox* cluster includes six *Hox* genes arranged in three pairs spread out over 5 Mb of chromosome III. *Ceh-13*, *lin-39*, *mab-5*, and *egl-5*, are organized in a loose cluster[43-45], while the other two genes *nob-1* and *php-3* are located more than 1 Mb away on the same chromosome[46-48].

Kenyon *et al*[42] reported that lin-39, mab-5 and egl-5 are required for postembryonic development. Emmons[49] showed that *mab-5* and *egl-5* are involved in cell fate specifications in males, and Sternberg[50] reported that *lin-39* is mainly involved in vulval cell fates and selects the outcome of Ras signaling (Figure 2). Gener-

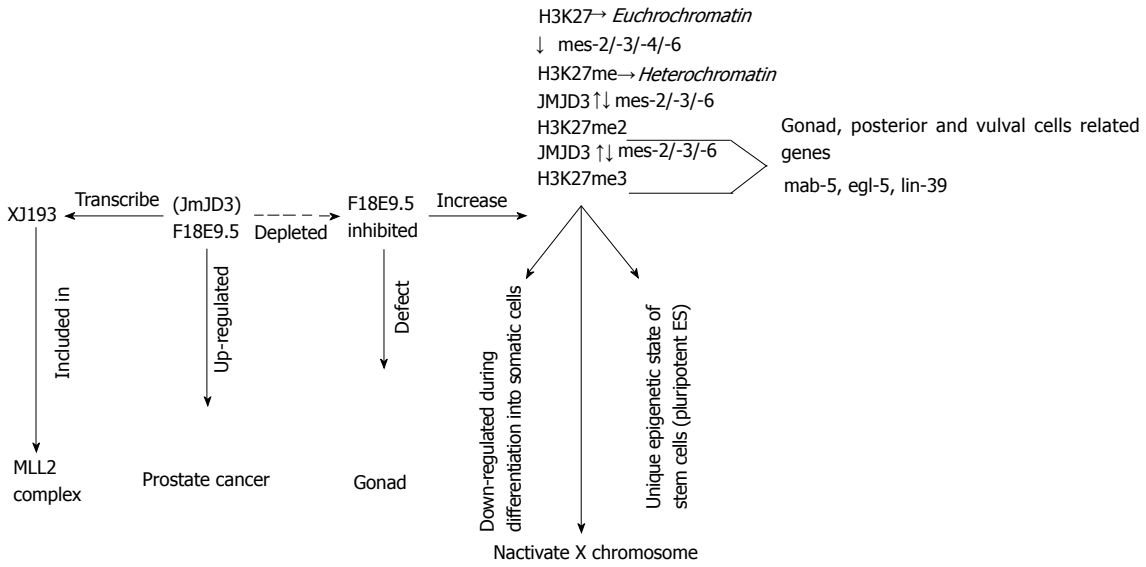

Figure 4 H3K27 modifications, Hox genes, and cellular development.

Table 4 Ubiquitin-conjugating enzymes in *Caenorhabditis elegans*: homologs and loss-of-function phenotypes

C. elegans	Peptide-conjugated	Human	Phenotypes
ubc-1	Ub	UBE2A; UBE2B	WT (RNAi)
ubc-2/let-70	Ub	UBE2D1/UBCH5A; UBE2D2/UBCH5B; UBE2D3/UBCH5C	embryonic arrest at pre-comma stage (RNAi)
ubc-3	Ub	CDC34; FLJ20419	WT (RNAi)
ubc-6	Ub	NCUBE1	WT (RNAi)
ubc-7	Ub	UBE2G1	WT (RNAi)
ubc-8	Ub	UBE2H	WT (RNAi)
ubc-9	SUMO	UBE2I	embryonic arrest post- gastrulation before muscle movement (RNAi)
ubc-12	NEDD-8 (Nedd8)	UBE2M	embryonic arrest at the comma stage (RNAi)
ubc-13	Ub	UBE2N; BAA93711	WT (RNAi)
ubc-14	Ub	UBE2G2	embryonic arrest post- gastrulation before muscle movement (RNAi)
ubc-15	Ub	NCUBE1	WT (RNAi)
ubc-18	Ub	UBE2L1; UBE2L3/UBCH7; UBE2L6	reduced growth rate and brood size (mut)
ubc-20	Ub	HIP2	impenetrant L3 and L4 larval arrest (RNAi)
ubc-21	Ub	HIP2	WT (RNAi)
ubc-22	Ub	UBE2L1; UBE2L3/UBCH7; UBE2L6	WT (RNAi)
ubc-23	Ub	HIP2	WT (RNAi)

ally, patterns of *Hox* gene expressions in *C. elegans* are controlled by cell lineage, which is completely different when compared to Drosophila or vertebrate systems[51]. However, as depicted in Figure 2, Wnt signaling pathways regulate some aspects of *Hox* gene expression during vulval development[52].

DNA methylation patterns are characterized by low tissue specificity when compared to other epigenetic information such as acetylation, phosphorylation, ubiquitylation, and ADP-ribosylation. These findings suggest that DNA methylation prediction is relatively easier to handle than other histone modifications[53]. In addition, *C. elegans* lacks cytosine methylation, and therefore it represents a potential candidate to explore for possible existence of an epigenetic code. This paper aims to investigate patterns of histone modifications in *C. elegans* that occur on Hox genes within a chromatin context. This investigation seeks to analyze the identified patterns and map histone modifications to the transcriptional status of specific *Hox* genes: histone code.

EXPLORING THE HISTONE CODE

Homeotic transformations that lead to body structure loss or duplication occur due to inappropriate expression of *Hox* genes. Thus, it is fundamental to identify a histone code that establishes a correlation between the histone modifications and a heritable histone code. In this work, only four *Hox* genes in *C. elegans* are investigated: *lin-39*, *mab-5*, *egl-5*, and *ceh-13*. It is known that each *C. elegans Hox* gene is expressed in restricted regions of multiple diverse tissues and lineally unrelated cells and defines the region specific differentiation characteristics[54].

Generally, *Hox* genes are globally repressed by the polycomb group (*PcG*) in mammals as well as in *C. elegans* (Figure 2). In addition, any mutations in *PcG* genes lead to ectopic *Hox* gene expression which will also in turn lead to posterior homeotic transformations in both Drosophila and vertebrates[55,56]. A further analysis of Drosophila *trxG/PcG* double mutants showed that activity of *TrxG/MLL* complexes is required to block *PcG*-mediated silencing of transcribed *Hox* genes. The *TrxG/MLL* complex that catalyzes the H3K4me3 is associated with active transcription[57,58]. Consequently, promoters of active genes develop to be enhanced with H3K4me3 modified nucleosomes. Similarly, in *C. elegans*, the *MLL2*

Table 5 Hox genes of the *Caenorhabditis elegans*: Homologs of humans

Gene product		Mammalian relative(s)	Molecular function
EGL-5	EGg-laying defects	Hox9-13 (all human posterior *Hox* genes)	Hox transcription factor. Upregulated by Ras signaling.
LIN-39	Abnormal cell LINeage	HoxB5	Hox transcription factor. Upregulated by Ras signaling.

complex plays the same role as in Drosophila (Figure 2).

Chromatins within mouse embryonic stem (mES) cells include M^2H3K4 and M^3H3K27 marks at *Hox* gene promoters, in which both repressive and activating chromatin modifications were referred to as "bivalent domains"[57]. These bivalent domains may lead *Hox* genes to an activation state. A challenging task is to determine how *Hox* genes become transcriptionally activated during ES cells differentiation or embryonic development.

Components of the *MLL2* complex in humans were shown to be initially recruited at the promoters of the most anterior *HOXA* and *HOXB* genes, with *H3K4* becoming tri-methylated[59]. The presence of UTX (Ubiquitously transcribed tetratricopeptide repeat, X chromosome, linked with histone dementhylation) with a simultaneous loss of *PRC2* and *H3K27me3* marks from the promoters resulted in a rapid activation of these genes. These findings suggest that UTX could be essential for activating *Hox* genes, since its loss of expression did lead to a strong decrease in *HOXB1* transcription. These findings are used later to support a particular hypothesis in conceiving the Histone code in *C. elegans*.

It has been reported in *C. elegans* that *mab-5* is expressed in the V5 and V6 cell lineages, which directs these cells to develop into rays. Further, *egl-5* is expressed only in the V6 lineage, which is required for the development and differentiation of V6 rays[60]. The normal development of the *C. elegans* male tails requires *SOP-2*, *MES-2/-3/-6* (*PcG* genes) and *lin-49/lin-59* (trithorax-related genes). As it is depicted in Figure 2, the misregulation of *mab-5* and *egl-5* is associated with the defects of ray development in *lin-49*, *lin-59*, *SOP-2*, and *MES*-mutants[61,62]. In *C. elegans* as well as in Drosophila, *PcG* proteins operate as transcription repressors, whereas trithorax proteins operate as transcription activators[63] .

Mutations in Hox genes lead to irregular patterns of programmed cell death. For example, in *C. elegans*, *mab-5* has been reported to be essential for the programmed cell deaths of two lineally related cells generated in the P11 and P12 lineages[64]. Further, Figure 2 shows that lin-39 was reported to control vulval cell development[65], and ceh-13 is required for development, and its ectopic expression during embryogenesis lead to embryonic lethality[66]. This latter is the orthologue of the Drosophila labial and the human *Hox1* genes.

Various parts of Figures 2-4 were constructed from exploring Wormbase (www.wormbase.org), which represents a major repository for *C. elegans* information. Figure 2 illustrates the fact that when *H3K4* is tri-methylated, it binds with the complex *lin-49/lin-59* which then expresses *mab-5* and *egl-5*. In normal cell development, *mab-5* and *lin-39* repress each other in turn. The loss of*H3K4me3* leads to X inactivation, whereas its increase occurs when rbr-2 (Jarid1 family) is inhibited, which then lead to a defect in vulval cells. However, it is not clear why *H3K4me3* binds with *JmJD2A*, and which proteins express or repress ceh-13, although its first expression is detected in the male tail from L3 until mid L4. In humans, components of the MLL2 complex were shown to be initially recruited at the promoters of the most anterior *Hox A* and *Hox B* genes, with *H3K4* becoming tri-methylated[59,67]. This finding suggests that *H3K4me3* could be involved in expressing *ceh-13*.

Figure 3 shows that all levels of methylated *H3K36* represent an activation mark and prevents transcriptional initiation downstream of the promoter region. This figure also shows a few correlative events. For example, *H3K36me3* expresses *lin-39*, whereas *H3K9me3* represses *lin-39*. The depletion of *JMJD2A* (H3K9/K36 demethylase) leads to an increase of *H3K9/K36me3* and to a *P53*-related apoptosis and an altered program of meiotic DSB repair. Like *H3K4me3*, *H3K9me3* has also been observed to bind with *JMJD2A*. However, no clue is available to comprehend the purpose of this binding.

Figure 4 illustrates the importance of H3K27me3 as it represents a unique epigenetic state of pluripotent ES cells, and it is mainly down-regulated during differentiation into somatic cells. Generally, all forms of methylated *H3K27* correspond to inactivation marks. However, *H3K27me3* is specifically involved in inactivating the X chromosome as well as *mab-5*, *egl-5*, and *lin-39*. The depletion of *F18E9.5* (member of JmJD3 family that demethylates *H3K27me3*) causes defects in gonadogenesis, whereas its up-regulation has been detected in prostate cancer[68] .

Figure 3 shows that the HMT met-2 catalyze H3K9 mono-, di- and tri-methylation in constitutive heterochromatin. The methylation of *H3K9* binds with the chromodomain of *hpl-2* in order to establish and maintain the heterochromatin structure.

Polycomb and trithorax groups are involved in maintaining the epigenetic code and the cell identity[69]. Polycomb complexes are found in closed chromatin structures and are thus involved in gene silencing, whereas trithorax complexes are found in open chromatin structure and are involved in maintaining active genes. Polycomb and trithorax groups are considered as HMTs and play a role in epigenetic inheritance[70,71]. Figure 4 depicts the mono-, di-, and tri- methylation of *H3K27* by the polycomb MES-2/-3/-6 proteins. In addition, it also shows that the di- and tri-methylation of *H3K27* are involved in repressing *mab-5*, *egl-5*, and *lin-39*. Further, Figure 2 shows that the trithorax complex *lin-49/lin-59* binds with *H3K4me3* and then activates *mab-5* and *egl-5*.

Figures 2-4 illustrate various paths that lead to expression and repression of *Hox* genes in *C. elegans*, and eventually help to describe the histone code: (1) *H3K4me3* is involved in activating *mab-5* and *egl-5*; (2)

H3K36me3 plays a role in activating *lin-39*; (3) *H3K9me3* is known in repressing *lin-39*; (4) *H3K27me2* and *H3K27me3* both repress *mab-5*, *egl-5*, and *lin-39*; and (5) although it is known that a high level of *pop-1* represses *ceh-13* and a low level of *pop-1* expresses it, nonetheless it is worthwhile investigating whether any histone modifications are involved in expressing or repressing it. Presently, evidential data only indicates that *H3K4me3* may perhaps express it. However experimental work is needed to support such a claim.

Histone acetylation is not yet demonstrated to be involved in epigenetic memory, since it is mainly a dynamic modification and is maintained by the ongoing activity of HATs and HDACs[21]. In addition, histone demethylase removes a methyl group from a particular histone tail. For example, *rbr-2* (Jarid1 family) demethylates *H3* at lysine 4 (Figure 2); JMJD2A protein demethylates *H3* at lysine 36 (Figure 2); and *F18E9.5* (*JmJD3* family) removes the tri-methyl group from *H3K27* (Figure 4). Correspondingly, other post-translationally modifications including phosphorylation, ubiquitylation, sumoylation, and ADP-ribosylation have not been shown to have an important role in epigenetic memory. Nevertheless, HATs and HDACs have been used in therapeutic targets in several diseases including cancer[72-75].

CONCLUSION

Histone modifications are clearly conserved within metazoans and correspond to a very ancient form of basal genetic regulation. Generally, each individual histone modification has the same biological effect in various organisms. For example, methylation of *H3K4* represents an activation mark in both humans and *C. elegans*. Evidently, the epigenetic code identified as the heritable transcriptionally states will contribute in biomedical research and particularly in epigenetic therapy. In addition, epigenetic regulation is shown to have a role in mental disorders, autoimmune diseases and many other complex diseases[76].

A number of silenced tumor suppressor genes are shown to be lost due to epigenetic deactivation rather than sequence damages, although epigenetic changes co-operate with genetic changes to initiate the development of a cancer since they are mitotically heritable[77,78]. Further, epigenetic irregularities are pharmacologically reversible as opposed to genomic damage[79]. This fact provides an incentive for the research community to devote more efforts to epigenetic therapy.

There is an on-going quest to discover drugs for diseases with genetic defects like cancer[80-83]. The purpose of investigating the histone code is to uncover the power of the epigenetic code and its use in drug design and discovery. Understanding the epigenetic machinery of the *Hox* genes and their cofactors could enable new targets for future therapies. As the investigation on Hox genes unravels, more translation to clinical application is expected. *Hox* genes have been used as biomarkers such as *HoxA9*[84], MLL translocation[85] and NUP98 fusions[86] in leukemias. In breast cancer, other groups have investigated the developed of a two-gene test using qRT-PCR to determine the ration of *HoxB13* expression to IL17RB expression that leads to predict the tumor recurrence in patients with eR-postive tumors taking tamoxifen[87,88].

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BRIEF ARTICLE

A prospective trial of volumetric intensity-modulated arc therapy *vs* conventional intensity modulated radiation therapy in advanced head and neck cancer

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Abstract

AIM: To prospectively compare volumetric intensity-modulated arc therapy (VMAT) and conventional intensity-modulated radiation therapy (IMRT) in coverage of planning target volumes and avoidance of multiple organs at risk (OARs) in patients undergoing definitive chemoradiotherapy for advanced (stage Ⅲ or Ⅳ) squamous cell cancer of the head and neck.

METHODS: Computed tomography scans of 20 patients with advanced tumors of the larynx, naso-, oro- and hypopharynx were prospectively planned using IMRT (7 field) and VMAT using two arcs. Calculated doses to planning target volume (PTV) and OAR were compared between IMRT and VMAT plans. Dose-volume histograms (DVH) were utilized to obtain calculated doses to PTV and OAR, including parotids, cochlea, spinal cord, brainstem, anterior tongue, pituitary and brachial plexus. DVH's for all structures were compared between IMRT and VMAT plans. In addition the plans were compared for dose conformity and homogeneity. The final treatment plan was chosen by the treating radiation oncologist.

RESULTS: VMAT was chosen as the ultimate plan in 18 of 20 patients (90%) because the plans were thought to be otherwise clinically equivalent. The IMRT plan was chosen in 2 of 20 patients because the VMAT plan produced concentric irradiation of the cord which was not overcome even with an avoidance structure. For all patients, VMAT plans had a lower number of average monitor units on average (MU = 542.85) than IMRT plans (MU = 1612.58) ($P < 0.001$). Using the conformity index (CI), defined as the 95% isodose volume divided by the PTV, the IMRT plan was more conformal with a lower conformity index (CI = 1.61) than the VMAT plan (CI = 2.00) ($P = 0.003$). Dose homogeneity, as measured by average standard deviation of dose distribution over the PTV, was not different with VMAT (1.45 Gy) or IMRT (1.73 Gy) ($P = 0.069$). There were no differences in sparing organs at risk.

CONCLUSION: In this prospective study, VMAT plans were chosen over IMRT 90% of the time. Compared to IMRT, VMAT plans used only one third of the MUs, had shorter treatment times, and similar sparing of OAR. Overall, VMAT provided similar dose homogeneity but less conformity in PTV irradiation compared to IMRT. This difference in conformity was not clinically significant.

Key words: Volumetric intensity-modulated arc therapy; Intensity-modulated radiation therapy; Target coverage; Organs at risk

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INTRODUCTION

Radiation therapy is a mainstay of treatment for both early and advanced stage head and neck cancer. Traditional head and neck conformal radiation therapy, in addition to problems related to matching of multiple beams, was often associated with multiple toxicities including xerostomia (dry mouth) dysgeusia, hearing loss, brain necrosis, osteonecrosis of the mandible. To minimize the difficulties of matching multiple beams and ameliorate toxicities, radiation therapy for most head and neck cancer has shifted away from traditional conformal techniques (3DCRT) to fixed-angle intensity modulated radiation therapy (IMRT). IMRT uses multiple intensity-modulated beams to deliver non-uniform dose to the target. Beam modulation is created using a multileaf collimator (MLC). Superimposing numerous small beams produces a dose distribution with better target dose conformity and better sparing of critical structures than 3DCRT. IMRT allows the ability to escalate the target volume dose while reducing the dose to surrounding normal tissue and sparing organs at risk (OAR). Disadvantages of fixed angle IMRT compared to conformal therapy include: longer radiation delivery time and increased patient exposure to low dose radiation.

Recently, a new version of IMRT, volumetric modulated arc therapy (VMAT) has been developed. In VMAT, instead of using multiple fixed fields, the radiation is delivered in a continuous arc as the linear accelerator rotates around the patient, while the beam is modulated via the MLC, variable dose rate and variable gantry speed. Early reports suggest that VMAT produces dose-distributions comparable to IMRT for a variety of treatment sites[1-5]. While retrospective dosimetric planning studies have compared the techniques in head and neck cancer[6-8], no prospective clinical study had been done. As part of the institutional quality assurance/quality improvement process, a prospective study comparing VMAT and IMRT plans for dose homogeneity, dose conformality and ability to spare OAR was performed among 20 consecutive patients with advanced (stage Ⅲ and Ⅳ) cancers of the head and neck. The clinically superior plan was selected for treatment by the treating radiation oncologist.

MATERIALS AND METHODS

Patient selection and computed tomography simulation

In 2009, 20 consecutive patients with advanced (stage Ⅲ and Ⅳ) head and neck tumors were selected for this prospective study. The study was approved by the institutional review board. Computed tomography (CT) simulation was performed with patients in the supine position, a neck cradle was used for support and Aquaplast facemask was custom fit for immobilization. CT imaging was performed from vertex to 2 cm below the clavicle in 2.5 mm-thick slices. Scans were transferred to the Eclipse treatment planning station for simultaneous planning of both IMRT and VMAT treatment plans.

Volume definition

The extent of the primary tumor volume was based on physical examination of the head and neck, review of video laryngoscopy, and review of available diagnostic imaging. Gross tumor volume (GTV) was defined as the primary tumor volume and involved cervical lymph nodes (based on enlargement by CT imaging or abnormal uptake of radiolabeled [18F]-2-fluoro-deoxy-D-glucose (FDG) on PET/CT imaging). All treatment plans were prescribed to at least two dose levels, a high dose (HD) and a lower, elective dose (ED). Clinical target volume receiving a high dose (CTV_{HD}) was defined as GTV plus a 1cm margin. Clinical target volume receiving an elective dose (CTV_{ED}) consisted of clinically-negative bilateral cervical lymph nodes at risk of metastatic disease plus a 3mm margin. In select cases, when the tumor was felt to be infiltrative (endophytic) or when the border was ill defined, an intermediate volume (CTV_{INT}) was defined slightly larger than CTV_{HD} to prescribe an intermediate dose between the high dose and elective dose. Planning target volumes (PTV_{HD}, PTV_{ED}, PTV_{INT}) were defined as respective clinical target volumes (CTV_{HD}, CTV_{ED}, CTV_{INT}) plus a 3 mm margin for setup errors. The uninvolved cervical lymph nodes at risk were contoured as defined by the Radiation Therapy Oncology Group (RTOG) consensus guidelines[9]. Segmented OAR were the parotid glands, spinal cord, brainstem, cerebellum, cochlea, brachial plexus, anterior half of tongue.

Dose prescription

For patients with unresected tumor, PTV_{HD} was prescribed to a total dose of 70 Gy in 35 fractions at 2 Gy per fraction. PTV_{ED} was prescribed 56 Gy in 35 fractions at 1.6 Gy per fraction. When indicated, PTV_{INT} was prescribed to anywhere between 60-66 Gy in 35 fractions at 1.7-1.9 Gy per fraction. For patients receiving post-operative radiation, the primary tumor bed and involved nodes (PTV_{HD}) was prescribed to a total dose of 66 Gy in 33 fractions at 2 Gy per fraction. PTV_{ED} was prescribed to 56 Gy in 33 fractions at 1.7 Gy per fraction. Constraints for volume coverage and dose limits for OAR used for IMRT and VMAT planning are described below.

Conventional IMRT and VMAT planning

IMRT plans were generated using 7-10 non-parallel, non-coplanar fields of 6MV photons using a dynamic or sliding window technique. Optimization and dose calculations were performed with Eclipse version 8.1. The

Table 1 Critical structure dose tolerances

Critical structure/organ at risk	Dose (Gy)
Ant tongue (1/2 or 1/3)	70
Brainstem	54
Brain - 50%	54
Brachial plexus	60
Spinal cord	45
Cochlea	30
Parotid – 50%	30

PTVs were reduced to 3 mm below the skin surface to avoid acute dermal toxicity[10]. For optimization, the objective was to achieve all of the PTV volumes to receive > 95% of the prescribed dose. Dose constraints for the OAR were as per policy of the RPCI Radiation Medicine department as guided by Emami *et al*[11] as shown in Table 1. After optimization, the dose calculation was performed in Eclipse with the PBC algorithm using a calculation grid of 2.5 mm. Each VMAT plan consisted of two full arcs (-179 to 180 degrees), one clockwise and the second in the counter-clockwise direction. Collimator angle was selected between 30 and 45 degrees to cover the entire PTV. Dosing objectives for the PTV and OAR were as described for IMRT planning. VMAT planning was performed in Eclipse version 8.5, using the AAA calculation algorithm, and the Progressive Resolution optimization algorithm.

Plan evaluation and selection

Calculated doses to planning target volume (PTV) and OAR were compared between IMRT and VMAT plans. Dose-volume histograms (DVH) were utilized to obtain calculated doses to PTV and OAR, including parotids, cochlea, spinal cord, brainstem, anterior tongue, pituitary and brachial plexus, and were compared between IMRT and VMAT plans. In addition the plans were compared for dose conformity and homogeneity. The conformity index (CI), defined by RTOG 90-05 as the 95% isodose volume divided by the PTV_{HD} was used to assess plan conformity[12-13]. Dose homogeneity was measured by the average standard deviation of dose distribution over the entire PTV_{HD}. Ultimately, the clinically superior treatment plan was selected by the radiation oncologist and the prescription dose was normalized an isodose selected by the radiation oncologist to after review of the dose coverage of the PTV.

RESULTS

Patient characteristics

All patients included in the study had stage Ⅲ or Ⅳ head and neck cancer. All but 1 of the patients had a squamous cell carcinoma of the head and neck. One patient had adenoid cystic carcinoma. Two of 20 patients had invasive disease or other high risk features that warranted the prescription of an intermediate dose to a clinically determined intermediate volume (PTV_{INT}) and the average prescribed dose to this volume was 61.5 Gy.

Table 2 Patient characteristics

Pt	Pathology/site/stage			HD	ID	ED	Plan
1	SCC	Oropharynx	T3N3	70		56	VMAT
2	SCC	Larynx	T4N0	70	63	56	VMAT
3	SCC	Oropharynx	T2N2A	70		60	VMAT
4	SCC	Larynx	T3N2B	66		66	VMAT
5	SCC	Larynx	T3N2C	70		56	VMAT
6	SCC	Oropharynx	T2N2B	70		56	VMAT
7	SCC	Oropharynx	T3N0	70		56	VMAT
8	SCC	Larynx	T4N2C	70		56	VMAT
9	SCC	Nasopharynx	T1N3	70	60	55	VMAT
10	SCC	Oropharynx	T1N1	66		56	VMAT
11	SCC	Larynx	T3N0	70		56	VMAT
12	Medullary	Thyroid	T4bN1b	66		66	VMAT
13	SCC	Oropharynx	T4N2C	70		63	VMAT
14	SCC	Oropharynx	T2N2C	70		56	VMAT
15	SCC	Hypopharynx	T2N3	70		56	VMAT
16	SCC	Oropharynx	T2N3	70		60	VMAT
17	SCC	Larynx	T3N0	70		56	IMRT
18	SCC	Oropharynx	T3N2C	70		56	IMRT
19	SCC	Oropharynx	T3N2B	70		56	VMAT
20	SCC	Oropharynx	T2N1	70		56	VMAT

SCC: Squamous cell carcinoma; HD: High dose; ID: Intermediate dose; ED: Elective dose. VMAT: Volumetric modulated arc therapy; IMRT: Intensity modulated radiation therapy.

This information is summarized in Table 2.

Plan comparison and selection

VMAT was chosen as the plan to deliver in 18 of 20 patients (90%) because the plans were deemed to be clinically superior or otherwise clinically equivalent. The IMRT plan was chosen in 2 of 20 patients because the VMAT plan produced concentric irradiation of the cord which was not overcome despite the use of a spinal cord avoidance structure. An example of a VMAT plan delivering concentric irradiation is shown in Figure 1. Table 3 summarizes the number of monitor units (MU) required by each beam or arc for each IMRT and VMAT plan respectively. For every patient, the VMAT plan had a lower number of monitor units when compared to the respective IMRT plan. Average VMAT MU = 542.85 *vs* IMRT MU = 1612.58 ($P < 0.001$). Utilizing the conformity index as a measure of plan conformity, a perfectly conformal plan is described as CI = 1. Therefore as the CI approaches 1 the plan is more conformal. In the 20 patients included in this study, the average conformity of the VMAT plans (CI = 2.00) were less conformal when compared to the average conformity of the IMRT plans (CI = 1.61). This finding was statistically significant ($P = 0.003$). Dose distribution over the PTV_{HD} was on average, more homogeneous in the VMAT plans (average standard deviation of PTV_{HD} dose = 1.45 Gy) when compared to the IMRT plans (average standard deviation of PTV_{HD} dose = 1.73 Gy). Figure 1 shows the DVH curves for PTV_{HD} for all patients. The mean IMRT and mean VMAT DVH curves are plotted against each other. As shown in this figure, the mean VMAT DVH has a more homogeneous dose compared to the

Table 3 Target volume dose delivery analysis of volumetric intensity-modulated arc therapy (two arcs) and intensity modulated radiation therapy (seven to ten sliding window fields) plans

	Monitor units		Conformity index		Dose Homogeneity	
Patient	VMAT	IMRT	VMAT	IMRT	VMAT	IMRT
1	431	2211	1.15	1.19	2.47	2.59
2	530	1264	2.69	1.85	1.23	1.78
3	465	1853	1.74	1.97	1.31	2.34
4	584	1681	1.34	1.35	1.61	2.33
5	526	1423	1.69	1.38	1.15	1.95
6	593	1560	2.00	1.43	1.07	2.01
7	552	2046	1.79	1.66	1.08	2.34
8	522	1579	1.34	1.28	2.06	2.66
9	672	1473	1.72	1.70	0.98	2.46
10	668	1359	1.90	1.42	2.38	1.91
11	614	1000	3.07	1.67	1.05	0.79
12	533	2047	1.27	1.38	1.92	1.42
13	483	2087	1.86	1.58	1.41	2.05
14	531	1319	1.65	1.23	1.27	2.06
15	544	1188	2.41	1.67	1.58	1.27
16	520	1769	2.50	2.43	1.69	1.06
17	519	1114	3.27	2.39	1.11	1.19
18	542	1273	1.63	1.64	1.31	0.81
19	565	1258	2.16	1.84	1.44	1.01
20	463	1135	3.95	2.26	0.98	0.58
Mean	542.85	1612.58	2.00	1.61	1.45	1.73
P value	< 0.001		0.003		0.069	

Comparison of monitor units required, conformity using conformity index and dose homogeneity using standard deviation of dose to PTV_{HD}. A lower CI corresponds to the more conformal plan. A lower standard deviation is seen in a more homogeneous plan. VMAT: Volumetric intensity-modulated arc therapy; IMRT: Intensity-modulated radiation therapy.

mean IMRT DVH (as the VMAT DVH is steeper than the IMRT DVH). This finding approached, but did not meet statistical significance ($P = 0.069$). Table 4 summarizes the mean dose (D_{mean}) to each of the studied organs at risk. Statistical significant difference was noted for the cochlea, however for all other critical structures there was no clear difference in mean dose. Overall, there were no differences in sparing organs at risk.

DISCUSSION

This study is the first prospective comparison of VMAT and IMRT in the actual treatment of advanced head and neck cancer patients. Ultimately, the VMAT plan was chosen for 18 of 20 (90%) patients. Compared to IMRT, VMAT plans used only one third of the MUs, had shorter treatment times, and similar sparing of OAR. Overall, VMAT plans trended towards better dose homogeneity but ultimately were found to have statistically significant less conformity in PTV irradiation compared to IMRT plans. This difference in conformality was not clinically significant.

In contrast to our prospective study which implemented the superior plan in the treatment of patients, all other reports comparing VMAT and IMRT for treatment planning in cancers of the head and neck have been retrospectively performed as theoretical exercises that were not intended to be directly implemented in

Table 4 Mean dose to organs at risk across all intensity modulated radiation therapy and volumetric intensity-modulated arc therapy plans

	Mean dose (Gy)		*P* value
	IMRT	VMAT	
Ant tongue	52.7	51.1	0.135
Brainstem	15.3	14.3	0.264
Left cochlea	23.7	18.8	0.002
Right cochlea	22.4	19.3	< 0.001
Left parotid	46.8	46.5	0.68
Right parotid	47.3	46.7	0.373
Spinal cord	20.4	20.9	0.173

VMAT: Volumetric intensity-modulated arc therapy; IMRT: Intensity-modulated radiation therapy.

patients. Overall, our results are consistent with the findings of several retrospective planning studies. Verbakel *et al*[7] found a statistically significant improvement in dose homogeneity with a similar compromise in conformity. However, unlike the findings reported here, Verbakel *et al* found an improved sparing of the parotid glands. A study by Alvarez-Moret *et al*[6] found comparable results between IMRT and double-arc VMAT in four patients. A third study by Bertelsen *et al*[8] compared IMRT to single-arc VMAT found no difference in dose homogeneity and equal or improved dose conformity with single-arc VMAT. Several of the metrics used in that study showed improved sparing of the parotids and spinal cord. The findings presented here show a significant improvement in MUs with VMAT using on average 66% of the MUs of the respective IMRT plan. A comparable reduction was shown by Verbakel *et al*[7] but not by Alvarez-Moret and Bertelsen. Some of these discrepancies may be explained by the fact that treatment planning was using the Eclipse planning system for a Varian linear accelerator in this study and that of Verbakel while the other two retrospective studies utilized Elekta systems.

The studies by Verbakel *et al*[7] and Alvarz-Monet *et al*[6]. compared IMRT plans with both single-arc and double-arc VMAT plans. Both reported that single-arc VMAT plans were inferior to double-arc plans and, unlike the findings of Bertelsen *et al*[8], single-arc VMAT plans were worse than IMRT. The double-arc plans were more comparable to IMRT plans, and as a consequence, single-arc plans were not included in this study.

In the past, some of the major issues raised with IMRT replacing the simpler 3D conformal RT plans were the more complicated treatment setup, and longer treatment times. However, the benefit to the patient in reducing xerostomia and other such side effects outweighed the drawbacks. With VMAT plans, treatment times are faster, beam-on times are shorter as evidenced by the lower number of monitor units on average with VMAT plans when compared to the IMRT plans. VMAT plans have less than a third of the number of monitor units as IMRT plans on average. This should decrease (though not eliminate) previous concerns about IMRT plans with higher monitor units leading to in-

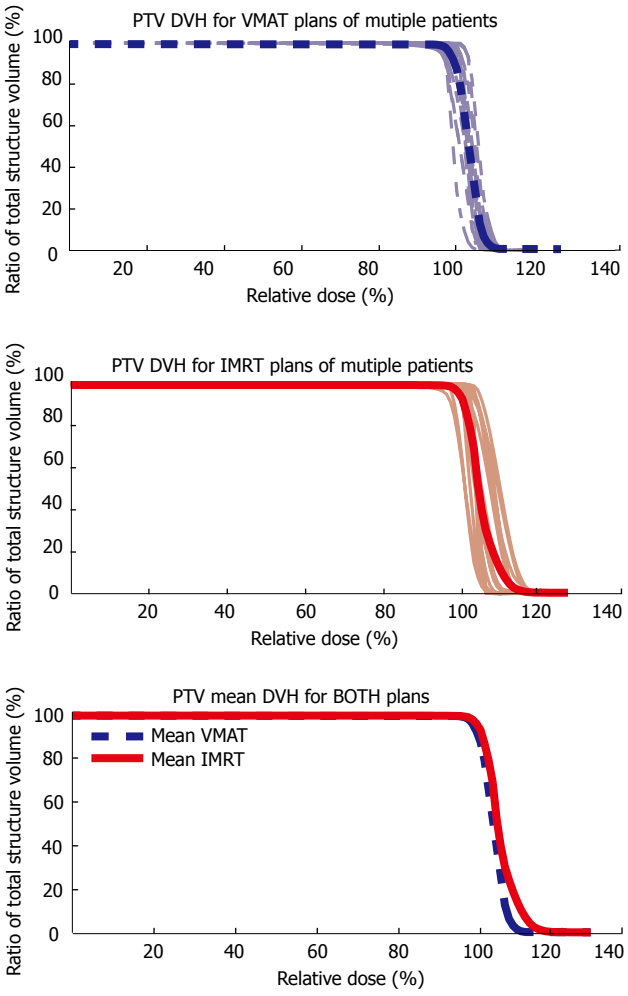

Figure 1 PTV$_{HD}$ dose volume histogram for all volumetric intensity-modulated arc therapy plans (blue), all intensity modulated radiation therapy plans (red). The mean intensity modulated radiation therapy dose volume histogram (DVH) (blue dashed line) is plotted on the same axis as the mean volumetric intensity-modulated arc therapy DVH (red solid line). PTV: Planning target volume; VMAT: Volumetric modulated arc therapy; IMRT: Intensity-modulated radiation therapy.

creased leakage radiation and increased risk of radiation induced second malignancies[14].

VMAT plans, on average, had a lower standard deviation of the dose delivered to the PTV$_{HD}$ when compared to the standard deviation of the IMRT plans. This is demonstrated by a steeper drop off in the DVH for the PTV$_{HD}$. The VMAT plans, by virtue of a lower standard deviation, and steeper drop off, had greater dose homogeneity when compared to the IMRT plans. This result trended towards but did not achieve statistical significance likely due to the small number of patients accumulated by this study. The ability to produce a more homogenous dose could be clinically relevant, by eliminating "cold spots" within the PTV, may improve not only primary tumor control but improve loco-regional control.

However when the plans were compared for conformity, IMRT was found to be more conformal by having a lower CI when compared to VMAT plans. A review of the conformity index by Feuvret *et al*[15] discussed the potential inaccuracies of the conformity index as defined by the RTOG, compared to other potential formulae to define conformity. The RTOG CI was used for this study, as it is the most commonly used measure seen in the literature, and as the simplest formula, it is the easiest to conceptualize. While the flaws inherent to using a single number to the similarity between two complicated 3D shapes (PTV$_{HD}$, 95% isodose volume) are obvious, the RTOG CI still provided a measure to compare the two plans. It is not clear whether a small loss of conformity between IMRT and VMAT planning is relevant to the overall clinical picture. In a review of the treatment plans, it was observed that for select patients there was spillage of the high dose well beyond the PTV$_{HD}$ and into the PTV$_{ED}$ as shown in Figure 2. This overflow of dose was not observed in the respective IMRT plan. Certainly this contributes to the higher CI of the VMAT plans when compared to the IMRT plans. At this time, it is not clear why or how the optimizer allows this overflow. This is an issue that warrants further study, the results of which will be published in future follow-up study.

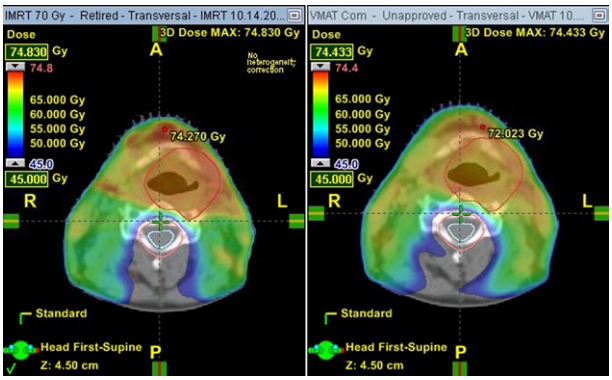

Figure 2 Example plan evaluation of intensity modulated radiation therapy *vs* volumetric intensity-modulated arc therapy for patient No.18. The volumetric intensity-modulated arc therapy plan (right) had dose that wrapped around the spinal cord despite an avoidance structure. The corresponding intensity modulated radiation therapy plan (left) did not.

There was no statistically significant difference in the mean dose delivered by both plans across most OAR that were studied. Only the cochlea (both left and right) demonstrated a statistically significant improvement in sparing dose to a critical structure with VMAT when compared to IMRT. The apparent loss in conformity as described earlier does not appear to worsen the ability of VMAT to spare critical structures when compared to IMRT. From this study, VMAT does not underperform IMRT in sparing OAR and produces plans that are comparable to IMRT in sparing OAR. Based on the findings of this study, and the improvements afforded by VMAT, currently all head and neck treatment plans are initially created using VMAT. Fixed-angle IMRT was performed only when the VMAT plan was found to be clinically unacceptable. More recently, this has become an increasingly rare event. Since this initial experience with VMAT, several techniques have been utilized at our institution to eliminate some of the issues found here,

and have resulted in an improvement in the conformity of the VMAT plans. These techniques will be utilized to retrospectively create new VMAT plans for the patients in this study and an update on this VMAT experience will be published in the near future.

In this prospective study, we set out to describe a single institution's initial clinical implementation experience with VMAT compared to current standard IMRT for advanced stage head and neck carcinomas. VMAT allowed faster treatment times and used 66% lower monitor units when compared to IMRT. Analysis of the treatment plans showed that VMAT plans were less conformal than IMRT plans. This is possibly due to spillage of higher dose outside of the PTV_{HD} and into the PTV_{ED}. The VMAT plans trended toward a more homogeneous dose, but did not meet statistical significance. OAR sparing by VMAT plans was comparable to IMRT plans. Ultimately, 90% of patients were treated with a VMAT plan that was either superior to, or comparable to its respective IMRT plan, as selected by the treating radiation oncologist.

COMMENTS

Background

Radiation is a fundamental aspect of definitive treatment for patients with cancers of the head and neck. In the head and neck, there are many important glands, muscles and organs in a very small space, often very close to the tumor.

Research frontiers

Volumetric intensity modulated arc therapy (VMAT) is a recent novel advancement in the way radiation therapy is planned on the computer and delivered by the linear accelerator.

Innovations and breakthroughs

Prior studies of patients with head and neck cancer have shown excellent local control and an ability to avoid over-dosing adjacent organs at risk with conventional intensity modulated radiation therapy (IMRT). However, each treatment with IMRT can take a very long time to deliver. One of the benefits of using VMAT to deliver radiation is the ability to deliver a treatment in a much shorter time than IMRT. Dose heterogeneity was comparable, as was the sparing of critical organs at risk while delivering a treatment in a much shorter time.

Applications

This study suggests that head and neck radiation treatments previously delivered with IMRT can be delivered with VMAT, with a clinically insignificant decrease in conformity, over a much shorter treatment time. The major advantage of treatment delivery time can have theoretical improvements in patient comfort, and decreasing the risk of radiation-induced second malignancy.

Peer review

Well written clinical study comparing IMRT and VMAT.

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CASE REPORT

A case of very large intrahepatic bile duct adenoma followed for 7 years

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Abstract

A 70-year-old man was referred to our hospital due to abnormal liver function. A tumor of 92 mm × 61 mm was detected on ultrasound screening of the left liver lobe. Although the tumor was suspected to be intrahepatic bile duct carcinoma, he had chronic heart disease and was unable to undergo surgery. Therefore, he was followed without further testing. No increase in tumor serum markers or tumor size was observed for the subsequent 7 years. We continued to suspect intrahepatic bile duct carcinoma, and we decided to perform a tumor biopsy. Tumor biopsy findings indicated intrahepatic bile duct adenoma (BDA), which is a rare benign epithelial liver tumor typically ranging from 1 mm to 20 mm. We herein report a case of very large BDA followed for 7 years.

Key words: Intrahepatic bile duct adenoma; Large tumor; Differential diagnosis; Benign liver tumor

Peer reviewer: Naoya Sakamoto, MD, PhD, Associate Professor, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

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INTRODUCTION

Intrahepatic bile duct adenoma (BDA) is a rare, benign epithelial liver tumor that typically ranges in size from 1 mm to 20 mm[1]. BDA is mainly found incidentally at laparotomy or autopsy[1-5]. Here, we report a case of very large BDA (about 90 mm in diameter) that was followed for 7 years.

CASE REPORT

A 70-year-old man was referred to our hospital due to liver dysfunction (Table 1). Although no liver failure was shown, mild increased serum ALP and γ-GTP were observed. A tumor of 92 mm × 61 mm was observed on ultrasound screening of the left liver lobe. Unenhanced abdominal computed tomography (CT) showed a tumor of the same size with dilation of the peripheral bile duct, while dynamic contrast-enhanced CT revealed heterogeneous enhancement in the venous phase (Figure 1). Although the tumor was suspected to be intrahepatic bile duct carcinoma, he had chronic heart disease and was unable to undergo surgery. Therefore, he was followed without further testing. No increase in serum tumor markers, such as CEA and CA19-9, or in tumor size was observed during the subsequent 7 years.

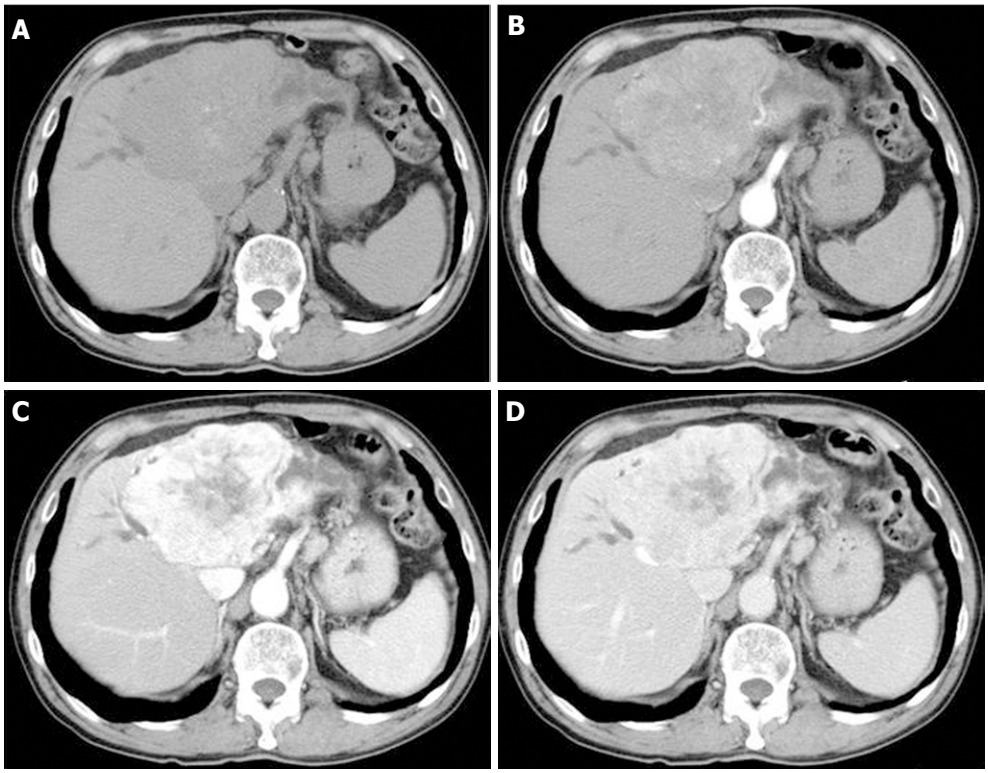

Figure 1 Unenhanced and enhanced computed tomography images. A: Plain; B: Arterial phase; C: Equilibrium phase; D: Venous phase. Enhanced abdominal computed tomography scan showed a tumor of 92 mm × 61 mm in the left liver lobe, with dilatation of the peripheral bile duct. In addition, tumor showed more heterogeneous enhancement in the venous phase.

Table 1 Laboratory data at initial hospitalization

WBC	7000/uL	BS	100 mg/dL
Neutro	60.9%	CRP	0.72 mg/dL
Eosin	3.6%	Na	139 mEq/L
Baso	0.5%	K	4.5 mEq/L
Mono	9.1%	Cl	103 mEq/L
Lympho	25.9%	CEA	2.5 ng/mL
RBC	413 × 10^4/uL	CA19-9	46 U/mL
Hb	12.8 g/dL	Alb	4.2 g/dL
Ht	41.2%	BUN	20.8 mg/dL
Plt	19.8 × 10^4/uL	Cr	1.07 mg/dL
TP	8.4 g/dL	D-Bil	0.1 mg/dL
T-Bil	0.9 mg/dL	ALT	12 IU/L
AST	22 IU/L	ALP	360 U/L
PT	56%	γ-GTP	168 IU/L
ChE	274 U/L		

WBC: White blood cell; BS: Blood suger; CRP: C-reactive protain; CEA: Carcinoembryonic antigen; BUN: Blood urea nitrogen ; ALT: Aspartate aminotransferase; ALP: Alkaline phosphatase; AST: Alanine aminotransferase; PT: Protronbin time; TP: Total protain; RBC: Red blood cell.

At that point, hepatic arterial angiography, abdominal CT angiography, gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced abdominal magnatic resonance image (MRI) and tumor biopsy were performed in order to confirm the diagnosis of intrahepatic bile duct carcinoma. Hepatic arterial angiography and CT angiography showed a hypervascular tumor of the left liver lobe with an enhancement effect persisting into the late phase, suggesting that this tumor had rich fibrous tissues (Figure 2). MRI confirmed that the tumor showed heterogeneous high intensity on T2-weighted images (Figure 3B) and low intensity on T1-weighted images (Figure 3A). Contrast-enhanced EOB MRI revealed that this tumor had relative hypointensity in comparison with the normal liver parenchyma on equilibrium and hepatobiliary phase (Figure 3C and D). Biopsy findings showed that the tumor consisted of small heterogeneous tubular ducts with fibrous tissues, without cell atypia or mitotic activity. Thus, diagnosis was confirmed as BDA (Figure 4).

DISCUSSION

Although the actual incidence of BDA is unknown, it is a rare, benign and asymptomatic tumor of the liver, and is typically found incidentally[1-5]. Most BDAs are subcapsular, ranging from 1 mm to 20 mm in diameter[1]. However, in the present case, the tumor was very large, at 90 mm in diameter. Therefore, we initially suspected tumor intrahepatic bile duct carcinoma, and believed the patient to be end-stage. Thus, we followed him without treatment over a 7-year follow-up period.

Although various abdominal imaging approaches have been reported, most BDAs show hypervascular characteristics consisting of prolonged enhancement[6-9]. Our case showed the same findings as in these reports, and it was suggested that the delayed or prolonged enhancement on dynamic CT and MRI was due to the fibrous

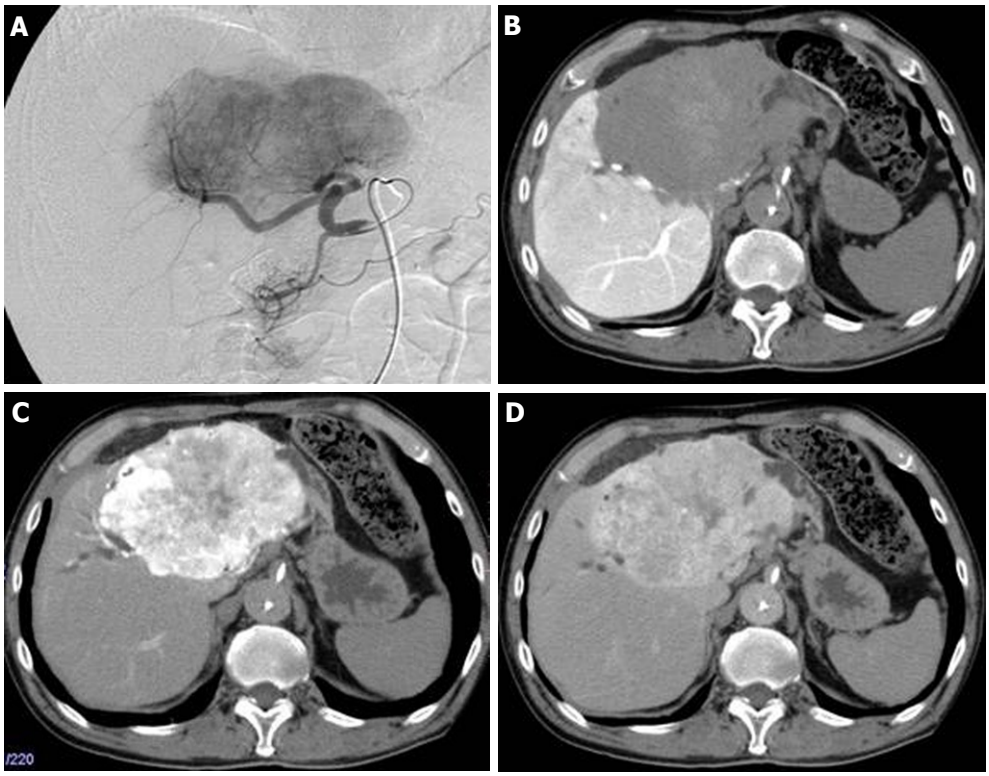

Figure 2 Follow-up hepatic arterial angiography and computed tomography images after 7 years. A: Hepatic arterial angiography showed hypervascular tumor of left liver lobe; B: Computed tomography (CT) angiography (portal phase) showed portal vascularity defect in the tumor; C-D: CT angiography (C: Arterial early phase; D: Arterial late phase) showed hypervascularity and a persistent enhancement effect.

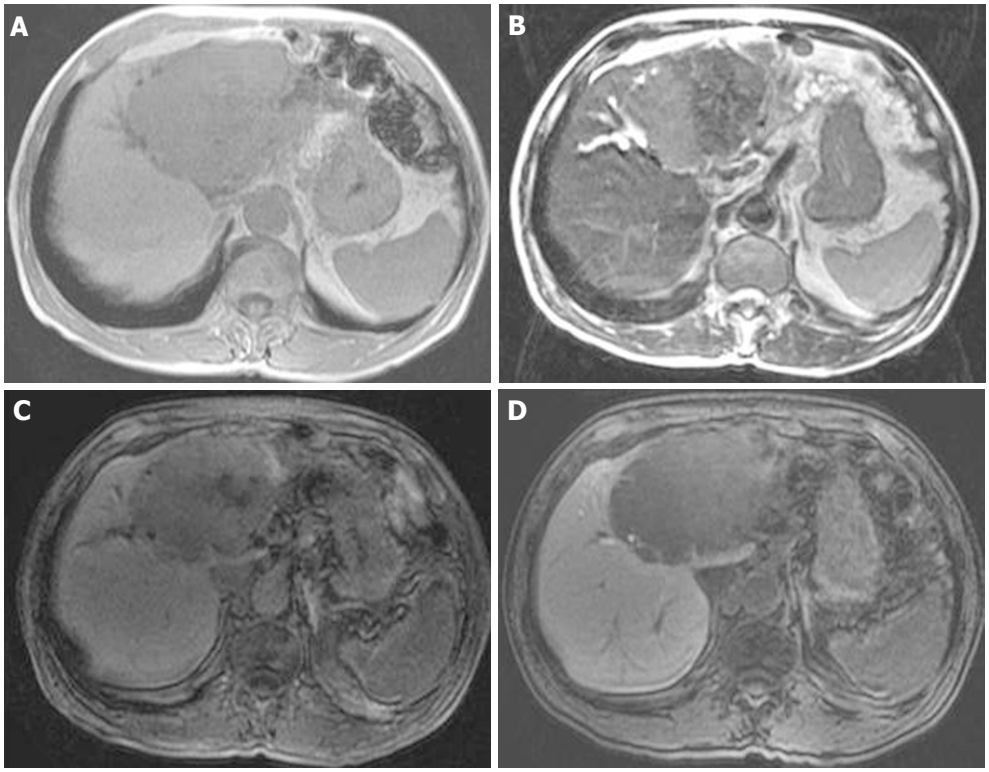

Figure 3 Follow-up magnatic resonance image images after 7 years. A: Large tumor of left lobe showed low intensity on T1-weighted images; B: Large tumor of left lobe showed heterogeneous high intensity on T2-weighted images; C-D: Large tumor of left lobe showed relative hypointensity in comparison with normal liver parenchyma on equilibrium and hepatobiliary phase of contrast-enhanced EOB magnatic resonance image.

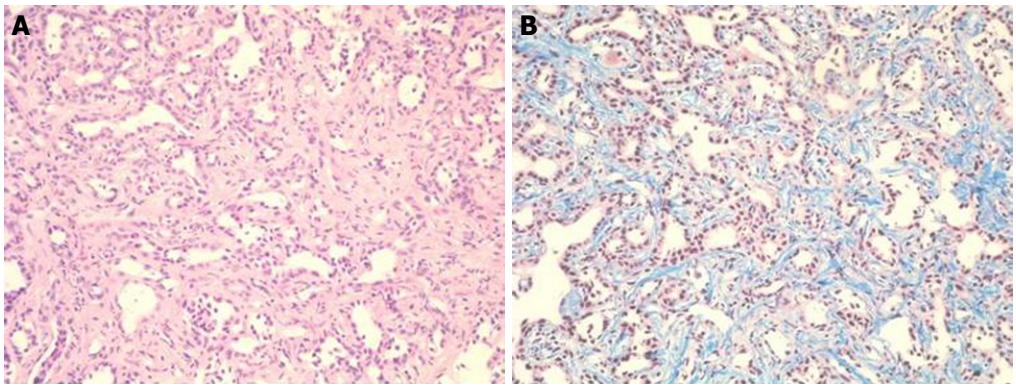

Figure 4 Tumor biopsy findings. Tumor consisted of small heterogeneous tubular ducts with fibrous tissues, without cell atypia or mitotic activity. A: Hematoxylin and eosin stain (× 400); B: Masson trichrome stain (× 400).

stroma within the tumor[8]. However, it has also been reported that the differential diagnosis between BDA and malignant tumor is very difficult using radiological methods[10]. In addition, although we obtained liver biopsy samples from some areas of the tumor, there may have been sampling errors due to the large tumor size and heterogeneous enhanced pattern. Therefore, we believe that this patient requires further follow-up.

To our knowledge, this is the first report of a very large BDA, and we confirmed no changes in size over a 7-year follow-up period. Although rare, BDA should be considered in the differential diagnosis of hepatic hypervascular tumors.

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January 16-17, 2012
Biomarkers Summit Egypt
London, United Kingdom

January 25-26, 2012
Multi-Disciplinary Appoaches to Cancer Therapy
Dubai, United Arab Emirates

January 26-27, 2012
3rd National Conference: Renal and Bladder Cancer 2012
London, United Kingdom

January 30-31, 2012
2nd Annual Clinical Trials in Oncology
Rome, Italy

February 2-3, 2012
Stem Cells 2012 Conference and Exhibition
San Diego, CA, United States

February 6-8, 2012
Mahidol International Conference on Infections and Cancers 2012
Bangkok, Thailand

February 12-17, 2012
Keystone Symposia: Cancer and Metabolism
Alberta, Canada

February 22-25, 2012
Excellence in Oncology
Istanbul, Turkey

March 8-10, 2012
10th International Congress on Targeted Anticancer Therapies
Amsterdam, Netherlands

March 9-10, 2012
13th European Congress: Perspectives in Lung Cancer
Amsterdam, Netherlands

March 14-16, 2012
BTOC-11 Biological Therapy of Cancer
Munich, Germany

March 15-17, 2012
3rd Conference on Therapeutic Resistance in Cancer
Quebec, Canada

March 29-30, 2012
Modern methods of diagnosis and treatment of malignant tumors
Kiev, Ukraine

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 20-21, 2012
Diagnosis and treatment of advanced forms of prostate cancer, bladder cancer and kidney cancer
Kiev, Ukraine

April 20-22, 2012
The 9th Meeting of Asian Society for Neuro-Oncology
Taipei, Taiwan

April 26-28, 2012
3rd International Video Workshop on Radical Surgery in Gynaecological Oncology
Prague, Czech Republic

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 5-6, 2012
Radiation Research Methods as A Diagnostic and Therapeutic Support in Oncology
Kiev, Ukraine

May 17-18, 2012
Eurasian forum on the management of patients with tumors of the gastrointestinal tract
Uman, Ukraine

June 16-17, 2012
Issues of Neurosurgery, vascular neurosurgery, neurooncology, spinal surgery and spinal cord
Kiev, Ukraine

July 7-10, 2012
22nd Biennial Congress of the European Association for Cancer Research
Barcelona, Spain

July 21-28, 2012
Cancer In Women
Hawaii, HI, United States

July 25-27, 2012
5th Latin American Conference on Lung Cancer
Rio de Janeiro, Brazil

August 27-30, 2012
UICC World Cancer Congress 2012
Québec, Canada

September 6-8, 2012
The 8th International Jordanian Oncology Society Conference
Amman, Jordan

September 27-28, 2012
Current issues of diagnosis and treatment of oncogynecology diseases
Ivano Frankivsk, Ukraine

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European Conference of Oncology Pharmacy
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London, United Kingdom

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Modern aspects of diagnosis and treatment of breast cancer
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Sydney, Australia

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24th EORTC-NCI-AACR Symposium on "Molecular Targets and Cancer Therapeutics"
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GENERAL INFORMATION

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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

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