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REVIEW

Molecular genetics of early-onset colorectal cancer

Olivia Marx, Marc Mankarious, Gregory Yochum

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Abstract

Early-onset colorectal cancer (EOCRC) has been rising in global prevalence and incidence over the past several decades. Environmental influences, including generational lifestyle changes and rising obesity, contribute to these increased rates. While the rise in EOCRC is best documented in western countries, it is seen throughout the world, although EOCRC may have distinct genetic mutations in patients of different ethnic backgrounds. Pathological and molecular characterizations show that EOCRC has a distinct presentation compared with later-onset colorectal cancer (LOCRC). Recent studies have identified DNA, RNA, and protein-level alterations unique to EOCRC, revealing much-needed biomarkers and potential novel therapeutic targets. Many molecular EOCRC studies have been performed with Caucasian and Asian EOCRC cohorts, however, studies of other ethnic backgrounds are limited. In addition, certain molecular characterizations that have been conducted for LOCRC have not yet been repeated in EOCRC, including high-throughput analyses of histone modifications, mRNA splicing, and proteomics on large cohorts. We propose that the complex relationship between cancer and aging should be considered when studying the molecular underpinnings of EOCRC. In this review, we summarize current EOCRC literature, focusing on sporadic molecular alterations in tumors, and their clinical implications. We conclude by discussing current challenges and future directions of EOCRC research efforts.

Key Words: Early-onset colorectal cancer; Later-onset colorectal cancer; Mutations; oncogenes; Molecular characteristics; Transcriptomics

Core Tip: Early-onset colorectal cancer (EOCRC) has a considerably different clinical presentation and genetic profile compared with later-onset colorectal cancer. Furthermore, molecular alterations in EOCRC tumors differ in patients from separate geographical locations and distinct ethnic groups. Small human cohorts and the lack of a suitable mouse model system limit EOCRC studies, however, several actionable clinical targets and biomarkers specific to EOCRC have been identified. In this review, we discuss molecular alterations in EOCRC tumors at the DNA, RNA, and protein levels, and suggest future work to examine how these changes contribute to EOCRC pathogenesis.

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INTRODUCTION

Early-onset colorectal cancer is a growing global issue

Cancers of the colon and rectum are the third most commonly found in both men and women globally [1]. Colon cancer screenings have increased early detection in patients over the age of 50-year-old and have contributed to the overall decline in global rates of colorectal cancer (CRC)[1]. However, the population of early-onset CRC (EOCRC) patients, under 50-year-old, has been steadily rising over the past several decades[2,3], and by 2030, the rates of early-onset colon and rectal cancers are expected to increase by 27.7%, and 46.0%, respectively[4]. Unfortunately, 10%-15% of CRC patients are diagnosed before the age of average-risk screening recommendation (before 2018, 50-year-old)[5]. Due to a lack of screenings and a delay in the diagnosis of younger patients, EOCRC is often detected at advanced stages, reducing the chances of long-term survival[6]. Many studies have shown that EOCRC is molecularly distinct from later-onset CRC (LOCRC), or CRC diagnosed after the age of 50-year-old. Compared with LOCRC, EOCRC has a differing frequency of oncogenic mutations[7], increased prevalence of mucinous and signet (poorly differentiated) histology[8], a more distal location[2], and exhibits a distinct DNA methylation profile[9]. Despite aggressive treatment of EOCRC patients, their overall survival is worse compared to those with LOCRC[5,10].

EOCRC risk factors

There is no clear cause for most EOCRC cases, although environmental risk factors are likely key contributors to cancer development. Lifestyle factors such as smoking, unhealthy diet, obesity, and alcohol consumption increase the risk of developing CRC early on[1]. In the United States, EOCRC has a strong birth cohort effect, implicating generational lifestyle changes in the development of EOCRC[11].

Several recent studies have demonstrated the association between obesity and metabolic disorders with the development of EOCRC[12,13]. Tang *et al*[14] found that EOCRC patients had a worse metabolic profile, with higher levels of triglycerides and lower levels of high-density lipoprotein cholesterol compared with LOCRC patients[14]. Molecular links between obesity, metabolic disorders, and CRC have been suggested, including the promotion of intestinal stem cell populations[15,16], increased insulin resistance, adipocyte levels, and inflammation[17]. How EOCRC risk factors affect clinical presentation is still under investigation. One aspect of EOCRC clinical presentation of particular interest is tumor location[18].

Differences in left- and right-sided EOCRC

Over half of pre-malignant polyps in EOCRC are found in the distal colon and rectum[18], and this has prompted calls for screening sigmoidoscopy at an earlier age than current guidelines, which were recently changed from 50 to 45[19]. While left-sided colon cancer is more predominant in EOCRC, right-sided EOCRC is associated with lower overall survival compared to left-sided EOCRC (44% *vs* 61%) [20]. Several factors have been implicated in the difference in survival between right-sided and left-sided CRC. During embryonic development, the proximal colon originates from the midgut while the distal colon originates from the hindgut. This developmental difference may impact cancer cell origins as well as the metastatic potential of tumors due to differences in vascularization. Additionally, several microbiota changes have been characterized between the proximal and distal colon which may play a role in oncogenesis[21,22]. Proximal colonic tumors also have distinct histopathological features as they tend to be more mucinous with microsatellite instability and mismatch repair (MMR) deficiency compared to distal tumors[23,24]. These distinctions may indicate unique molecular drivers of distal and proximal EOCRC tumors.

Common molecular drivers of colorectal adenocarcinoma

The multi-step progression from normal colonic mucosa to adenoma to CRC was first described in 1990 by Fearon and Vogelstein[25]. In this model, an *APC*-inactivating mutation is an initiating event, followed by *KRAS*-activating mutations driving adenoma development[25]. Further studies found that the malignant transformation of adenomas was driven by additional mutations in the tumor growth factor beta, *PIK3CA*, and *TP53* pathways (Figure 1)[26-28]. Sessile serrated polyps act as a precursor to up to one-third of CRCs and are thought to arise through mechanisms distinct from canonical *APC* mutation-driven polyps[29-31]. Instead, serrated polyps are thought to develop from a *BRAF* mutation and are also often characterized by DNA hypermethylation[29]. Mutations to *BRAF*, *KRAS*, and *TP53* are also found in other cancers and the multi-step progression to adenocarcinoma may not strictly follow the canonical or serrated pathways described. While most sporadic LOCRCs can be categorized by deregulation of canonical Wnt/β-catenin/*APC* signaling or serrated *BRAF* mutation pathways[30] (Figure 1), it is less clear how sporadic EOCRCs develop.

CRC is often categorized into consensus molecular subtypes (CMS). At the genomic level, CRC can be categorized as microsatellite instable (MSI, often caused by a defect in MMR genes) or chromosome instable. At the transcriptomic level, there are four main CMS. CMS1 is associated with immune JAK-STAT activation, microsatellite instability, and hypermutated tumor DNA[32]. CMS2 is associated with canonical Wnt/*MYC* activation, CMS3 is characterized by metabolic alterations, and CMS4 is associated with epithelial-mesenchymal transition and immunosuppression[32]. CMS1 tumors are more often considered CpG island methylator phenotype-high (CIMP-high, characterized by genome-wide hypermethylation). These CMS1 tumors also often have *BRAF(V600E)* mutations and are associated with sessile serrated adenomas[32]. *RSPO* fusions and *RNF43* mutations are often seen in hypermutated CMS1 CRC[32], and Yan *et al*[33] also identified these alterations in a subset of EOCRC tumoroids[33].

Differing frequencies of CMS in EOCRCs compared with LOCRCs have been identified. Willauer *et al*[34] showed that patients under 40 were more likely to exhibit CMS1 or CMS2, with CMS1 being considerably more prevalent in EOCRC compared with the average of most CRCs, though no differentiation between sporadic and hereditary mutations was made[34]. Therefore, the increased prevalence of Lynch syndrome, a hereditary MMR deficiency syndrome, in younger patients[7,35] could contribute to this observation. In fact, MSI/CIMP-high tumors were associated with Lynch syndrome in young patients, whereas in older patients, they were associated with *BRAF* mutations[36]. Sporadic EOCRC patients are less likely to have Lynch syndrome[37] and are less likely to have tumors with a CpG island methylator phenotype[38], which are associated with CMS1[38]. Limitations to sample size necessitate future studies to examine the CMS of sporadic EOCRCs compared with LOCRCs.

Many excellent reviews explain the molecular subtypes and mutations in CRC[30,32,39]. Likewise, many excellent reviews have focused on the different clinical presentations and outcomes of EOCRC and LOCRC patients, with some mention of prevalent molecular distinctions of EOCRC[2,4,40]. However, reviews that focus primarily on the molecular characterizations of EOCRCs are limited. There has been a rise in EOCRC literature over the past 5 years that we aimed to summarize here. In this review, we searched the literature for early- and young-onset CRC, along with specific searches for molecular genetics, DNA methylation, histone alterations, transcriptomics, splicing, proteomics, ethnic disparities, and biomarkers. Relevant papers were selected for discussion. Here, we summarize key findings of the molecular genetic underpinnings of EOCRC thus far.

GENETIC STUDIES OF EOCRC

DNA mutations associated with EOCRC

Hereditary mutations often lead to CRC at a younger age in comparison with sporadic mutations. Compared with LOCRC, EOCRC patients have an increased polygenic risk score based on profiling single nucleotide polymorphisms[41]. Although approximately 30% of EOCRC cases report a family history of related cancers, only an estimated 10%-20% have known genetic risk factors like familial adenomatous polyposis, Lynch syndrome, or inflammatory bowel disease[7,10,42,43]. Therefore, our understanding of the genetic and molecular pathways that drive carcinogenesis in most patients is far from complete.

Sporadic EOCRCs, patients with no family history, have been shown to have different mutational profiles compared with LOCRCs (Table 1). Notably, many studies have found a significant decrease in the prevalence of *APC* and Wnt pathway mutations in EOCRC compared with LOCRC[33,43-45], with the exception of the β-catenin gene, *CTNNB1*, which is mutated in more EOCRCs compared with LOCRCs (Figure 2)[33,34]. Interestingly, a recent study by Yan *et al*[33] used organoids to demonstrate the heterogeneity between EOCRC patients, identifying some with *APC* mutations and others with *RSPO* fusions, which render the cultures hypersensitive to Wnt withdrawal[33].

MYC is a key oncogenic target of the Wnt/β-catenin pathway that is often deregulated in CRC[46]. Copy number variations of *MYC* are seen in 8%-15% of CRCs[47-49], however, we recently reported that 35% of EOCRC tumors from a 21-patient cohort had an increase in *MYC* copy number[50]. In addition, Pan *et al*[51] reported increased *MYC* copy number in younger CRC patients[51], however, another

Table 1 Differences in early-onset colorectal cancer and later-onset colorectal cancer DNA mutations

Gene	Prevalence in EOCRC *vs* LOCRC[1]	Role in cancer
APC	Decreased[33,34,44,45]	Blocks β-catenin, tumor suppressor
CTNNB1	Increased[33,34]	β-catenin, potentiates Wnt signaling, proliferation, and stemness
RNF43	Increased[43]/NS[33]	E3 ligase, negative regulator of Wnt signaling
BRCA2	Increased[54]	Double stranded DNA repair, tumor suppressor
PHLPP1	Increased[54]	Promotes apoptosis, inhibits *AKT*
TOPORS	Increased[54]	Regulates *TP53* stability, likely tumor suppressor
ATR	Increased[54]	*PI3/PI4* kinase, activates checkpoint proteins
MYCBP2	Increased[54]	*MYC* binding protein, activates *MYC*
FBXW7	Increased[44,54]	Ubiquitin ligase component, ubiquitinates *MYC*
POLE	Increased[44,54]	DNA polymerase E subunit, proofreading and DNA repair
BRAF	Decreased[34,38,57]/increased[43]	Proto-oncogene, activates *MAPK* signaling
TP53	Decreased[38,52]	Cell cycle inhibitor, tumor suppressor
NOMO1	Increased[53,126]	Inhibits nodal signaling. Deletion increases CRC cell migration
MYC	Increased[50,51]/NS[43,47]	Proto-oncogenic transcription factor, promotes proliferation and stemness
DNMT3B	Decreased[43]	De-novo DNA methyltransferase
MET	Decreased[43]	Proto-oncogene, promotes cell growth and survival
PTEN	Increased[57]	Tumor suppressor, negatively regulates *AKT* signaling
KRAS	Decreased[99,120]	Proto-oncogene, activates oncogenic signaling pathways

[1]Increase or decrease in the prevalence of genomic mutations or copy number variations in early-onset colorectal cancer compared with later-onset colorectal cancer tumors.
CRC: Colorectal cancer; NS: Not significant.

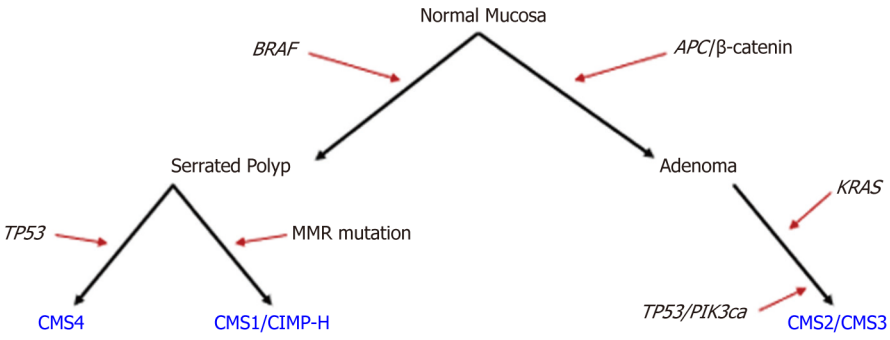

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Figure 1 Progression of normal mucosa to colorectal cancer subtypes. Shown are the major mutations in genes or pathways that have been implicated in the change from a normal colonic mucosa to cancer. In blue are the consensus molecular subtypes of cancers that arise from the preceding mutations based on transcriptomic analyses of colorectal cancer. This flow chart was assembled and modified from figures and information published in Langner *et al*[29] and Nguyen *et al*[31]. CMS: Consensus molecular subtype; MMR: Mismatch repair; CIMP: CpG island methylator phenotype-high.

study found no association between *MYC* copy number and age[47]. Overall, chromosomal deletions and copy number variations have been shown to be more common in EOCRC tumors compared with LOCRC tumors[52,53]. Alterations to *MYC* regulatory genes have also been identified in EOCRC, including *MYCBP2*[54], an E3 ubiquitin ligase that may regulate *MYC* transcription[55], and *FBXW7*[44,54], a tumor suppressive ubiquitin ligase that mediates degradation of *MYC*, among other oncoproteins, though more work is needed to determine the functional effect of these mutations[56]. Together, these findings provide evidence for a Wnt-independent increase in *MYC* activity in EOCRC.

In addition to differences in the prevalence of Wnt pathway/*MYC* mutations, many studies reported a decrease in *BRAF (V600E)* mutations (Table 1)[34,38,57], although Xu *et al*[43] noted an increase in *BRAF (V600E)* in EOCRC compared with LOCRC tumors[43]. This finding has clinical implications, as

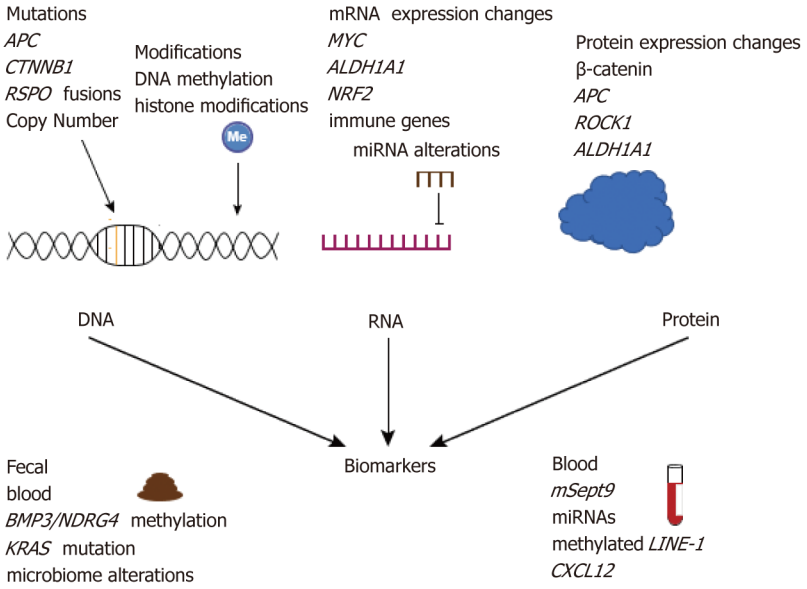

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Figure 2 Summary of key DNA, RNA, and protein alterations identified in early-onset colorectal cancer. Shown are DNA mutations and modifications, mRNA expression changes, and protein expression changes that have been reported to contribute to early-onset colorectal cancer and that may serve as biomarkers. miRNA: microRNAs.

BRAF (V600E) mutant tumors are associated with CIMP-high status, have a worse prognosis, and respond differently to cancer treatments[58]. The decrease in *APC* and *BRAF* mutations in EOCRC indicates that a higher percentage of EOCRC tumors may not follow the canonical or serrated carcinogenesis pathways commonly observed in LOCRC (Figure 1)[34]. Overall, many key oncogenes and tumor suppressors are differentially mutated in EOCRC compared with LOCRC, which may impact cancer progression and prognosis (Table 1, Figure 1). As new mutations unique to EOCRC are uncovered, more work is needed to determine how such mutations affect gene activity.

Epigenetic modifications in EOCRC

In addition to DNA mutations, studies have suggested that EOCRC has a distinct DNA methylation profile from LOCRC[9,59]. DNA methylation regulates gene expression and has been implicated in CRC [60]. Methylation of the long non-coding RNA *LINE-1* is often thought to represent global DNA methylation[61]. Studies suggest that DNA is overall hypomethylated in EOCRC compared with intermediate or LOCRC[42,59]. Epigenetic modifications may also be detectable in the blood, and a study by Walters *et al*[62] found hypermethylation of DNA repetitive elements, including *LINE-1*, in white blood cells from EOCRC patients[62]. While previous studies examined global DNA methylation, a recent high-throughput study by Joo *et al*[9] identified 234 differentially methylated regions unique to EOCRC tumors[9]. The authors then compared EOCRC DNA methylation patterns to those which occur upon age-related methylomic drift in the normal mucosa. They suggest that EOCRC tumors more rapidly accumulate cancer-related methylomic drift compared to intermediate or LOCRC tumors, though it remains unclear when this drift occurs during cancer progression[9]. More work is needed to assess DNA methylation over time and within patient-matched tumors and normal mucosa to better understand how age-related DNA methylation changes contribute to EOCRC.

In addition to DNA methylation, histone methylation and acetylation are associated with both aging and CRC[60], however, there have been few studies on histone modifications in EOCRC. A study in 2015 found that high levels of *H3K27me3* were associated with a better prognosis in younger CRC patients and a worse prognosis in older CRC patients[63]. DNA and histone modifications are a natural part of aging, however, how they impact gene expression, cancer progression, and drug response remains to be elucidated.

EOCRC transcriptomics

Transcriptome analysis is a comprehensive tool to identify deregulated signaling pathways in cancer[64, 65]. When applied to human tissues, this approach considers both genetic and environmental factors that contribute to the profile of deregulated gene expression on a per-patient basis. Several studies have analyzed the transcriptomic profile of EOCRC; however, many studies are limited by sample size and availability of patient-matched control samples.

Deregulation of mRNA targets of the Wnt/β-catenin pathway has been demonstrated in EOCRC, though at a lower frequency compared with LOCRC[33]. We have recently published transcriptome

analyses implicating deregulated *MYC*, and its downstream targets, in EOCRC[50]. The proto-oncogene *MYC* is upregulated in the intestines of obese individuals[66] and has been suggested to control obesity-mediated metabolic dysfunction in the intestines[67], though Ellegaard *et al*[68] found no relationship between *MYC* expression and body mass index[68]. Interestingly, our recent transcriptomic study found increased *MYC* expression in the EOCRC tumors of a subset of patients who were obese, suggesting a distinct tumor gene expression profile in obese and non-obese patients[50]. We did not find significant deregulation of the Wnt/β-catenin hallmarks of cancer in our EOCRC tumors compared with adjacent normal tissue. The age-associated role of *MYC* in CRC is supported by another recent study that implicated overexpression of *MYC*, along with the lncRNA *WiNTRLINC1* and the gene *ASCL2*, in younger colon cancer patients[69].

In addition to *MYC*, studies comparing EOCRC and LOCRC have found enrichment of cell signaling, apoptosis/inflammation, proliferation, adhesion, and development[38,70]. The recent success in cancer immunotherapies has prompted an interest in examining the immune profiles of CRC[71], however, few studies have interrogated the immune response in EOCRC. A recent study highlights the importance of aging and tumor immune response, showing that aging-related gene ontology sets were enriched in CRC tissues compared with normal tissues and this signature was higher in tumors with high immune infiltration[72]. Profiling approximately 40 tumors from both late- and early-onset CRC patients, Gardner *et al*[73] found that three immune genes *SAA1*, *C7*, and *CFD*, have deregulated expression in EOCRC primary tumors compared with LOCRC tumors[73]. Changes in the expression of these genes were shown to alter the tumor immune microenvironment and are associated with intestinal inflammation[73]. Another study identified age-associated changes in tumors compared with normal tissues and found enrichment of the nuclear factor erythroid 2-like 2 oxidative stress response in the tumors of younger patients compared with older patients (Figure 2)[74]. The tumor immune microenvironment is a complex system that involves many different cell types. Immune studies in EOCRC are limited by using a homogenized tumor population for bulk RNA sequencing instead of examining alterations at a single cell level.

Most transcriptomic studies of EOCRC have focused on mRNA, however, there is increasing evidence for the relevance of microRNAs (miRNAs) in cancer. miRNAs are short RNA transcripts that generally function to bind and repress a specific target mRNA. Two notable miRNA studies have been performed for EOCRC, the first by Nakamura *et al*[75] examined miRNAs from tumors and normal samples and found a seven-miRNA panel that was upregulated in EOCRC (n = 42), but not LOCRC (n = 370), in tumor *vs* normal tissues (Table 2)[75]. An earlier study using microarray analyses of a Turkish EOCRC cohort identified downregulation of *miR-143*, *miR-125b,* and upregulation of *miR-106a* in tumors *vs* normal tissues, although no comparison with LOCRC was performed[76]. While these miRNAs have been suggested as biomarkers for EOCRC, limited sample sizes, lack of patient-matched controls, and a lack of functional studies leave their roles in cancer progression unclear.

As with most EOCRC studies, transcriptomic analyses of EOCRC are limited by sample size and availability of quality sequencing data from tumors and patient-matched normal control samples. In addition to changes in transcript abundance, RNA sequencing can provide information about alternative polyadenylation and splicing events, which can alter protein structure and function. Alternative polyadenylation is associated with cellular proliferation and cancer[77]. It serves to alter the *3'UTR* length, which can affect miRNA regulation in many cancers including CRC[78]. Unfortunately, to our knowledge, there have been no studies on alternative splicing or polyadenylation events in EOCRC. However, one study did find a *POLE* mutation that may be associated with aberrant splicing in EOCRC [79]. As aberrant alternative splicing has been implicated in both CRC[80] and aging[81], we propose that examining EOCRC-specific splicing events would uncover novel insight into disease pathogenesis. Additional post-transcriptional modifications to mRNA, lncRNA, tRNA, and rRNA, such as methylation, have been associated with CRC but remain unexplored in EOCRC[82,83].

In addition to post-translational modifications and miRNA analysis, another type of transcriptomic analysis that is gaining popularity is single-cell RNA-sequencing (scRNA-seq), which can be used to identify gene regulation in the different cell types involved in cancer. Single-cell transcriptomics has been used to analyze the age-associated transcriptome in cancers including CRC[84]. Saul and Kosinsky [84] found that many aging- and senescence-associated genes were generally upregulated in cancers, including CRC[84]. These authors also found that CRC displayed distinct populations of epithelial cells with elevated age-related gene expression, underscoring the importance of examining age-related differences in CRC at a single-cell level[84]. Understanding immune infiltration and stem cell populations is crucial for developing cancer treatments that reduce the risk of relapse. Yan *et al*[33] performed scRNA-seq of EOCRC organoids and showed differing stem cell populations in response to Wnt media supplementation for six different EOCRC and LOCRC tumoroids with different underlying mutations[33]. Expanding scRNA-seq of EOCRCs would further elucidate information about disease progression that is specific to distinct cell populations in younger patients.

EOCRC proteomics

Proteomic studies have advanced cancer treatments by identifying therapeutic targets[85]. While the proteomic signature of CRC has been established[85,86], few studies have profiled the proteome of EOCRC[74,87]. Gong *et al*[87] recently published a paper using mass spectrometry to identify age-

Table 2 Differentially expressed transcripts in early-onset colorectal cancer

Gene(s)	Description
MYC	Proto-oncogenic transcription factor, increased in EOCRC tumors *vs* normal samples[50,69]
ASCL2	Transcription factor that promotes intestinal stem cells, increased expression in younger CRC[69]
ALDH1A1	Protein involved in cancer cell stemness, expressed higher in EOCRC tumors[88]
PEG10	Promotes proliferation and invasion, increased in EOCRC tumor *vs* normal and EOCRC *vs* LOCRC[70]
miR-143, miR-125b	miRNAs, under-expressed in EOCRC tumor *vs* normal[76]
miR-106a	miRNA, overexpressed in EOCRC tumor *vs* normal[76]
hsa-miR-4304, hsa-miR-513a-5p, hsa-miR-628-3p, hsa-miR-194-3p, hsa-miR-193a-5p, hsa-miR-210, and hsa-miR-4453	miRNAs uniquely overexpressed in EOCRC compared with LOCRC and normal tissues[75]
SAA1, C7, CFD	Immune genes differentially expressed in EOCRC *vs* LOCRC tumors[73]
NRF2	Protein involved in oxidative stress and inflammation, expressed higher in EOCRC *vs* LOCRC[74]

CRC: Colorectal cancer; EOCRC: Early-onset colorectal cancer; LOCRC: Later-onset colorectal cancer; miRNA: microRNAs.

associated differential expression of proteins in tumors compared to adjacent normal tissues. The authors found an age-associated proteomic signature in CRC tumors, which included *MYC*, *E2F*, and *mTORC1* targets, and proteins controlling the *G2M* checkpoint, DNA repair, and unfolded protein response (UPR) pathways expressed at higher levels in older CRC patients. Overall, 208 proteins were found to positively correlate with age, and only 20 negatively correlated with age. Many of these proteins reside in pathways that are targetable with known cancer drugs, supporting the potential use of specific cancer treatments for different ages of CRC patients[87]. For example, the proteins *PIN1*, *ROCK1*, and *ANXA5* are expressed higher in EOCRC and are targetable by Food and Drug Administration (FDA)-approved drugs or drugs in clinical trials[87]. While this study demonstrated the difference in proteomic signatures in younger and older CRC tumors, it was limited by the sample size of approximately 50 total patients with young, intermediate, or older onset CRC[87].

Another recent study by Holowatyj *et al*[74] found no significant differences (FDR q-value < 0.05) in the plasma proteome of younger-onset (n = 11) compared with older-onset (n = 45) CRCs using an antibody microarray platform to detect 206 inflammatory proteins. An increased sample size may shed light on interesting targets, as the authors found that the cancer-related proteins *BRCA2*, *PTEN*, *WNT5B*, and *WNT7A*, among others, had a fold change around two (P < 0.05) in EOCRC *vs* LOCRC serum[74]. While to our knowledge, no other proteome-wide studies have assessed EOCRC, some studies have identified individual proteins that are uniquely expressed in EOCRC tumors. For example, overexpression of the *ALDH1/ALDH1A1* protein has been identified in most EOCRC tumors compared with LOCRCs (Figure 2)[88], and an increase of β-catenin in the nucleus and cytoplasm of EOCRC compared with more membrane staining in LOCRC was shown *via* immunostaining[38].

Protein modifications such as glycosylation[89], ubiquitination[90], phosphorylation, and acetylation [91] are associated with CRC, but age-related characterizations remain limited. A recent study found that an increase in glycosylated hemoglobin in the serum of younger non-diabetic adults correlated with an increased risk for CRC[92], though no studies could be found that focused on post-translational modifications within EOCRC tumors. In addition to changes in protein modifications and expression changes, disruptions to protein folding are common in cancers, eliciting the UPR, which promotes cancer cell survival[93]. Indeed, our previous work showed enrichment of the UPR gene set in EOCRC tumors compared with adjacent control samples[50].

EOCRC in non-western countries

While many studies focus on profiling EOCRC in western countries, the incidence of EOCRC is also increasing in many Asian countries or regions such as Korea, Thailand, Japan, India, and Hong Kong [94,95]. While India reports one of the lowest rates of CRC incidence in the world[94], over half of the sporadic rectal cancers in this country are diagnosed in patients under 50-year-old[96-98]. In addition, studies from Indian cohorts found that under half of early-onset sporadic rectal cancer (EOSRC) tumors exhibit a Wnt signature, the most common driver of CRC, indicating distinct tumor drivers in this population[97,99]. Tumors without Wnt signaling showed increased activation of calcium/nuclear factor of activated T-cell signaling compared to EOSRC with high Wnt signaling[97]. Molecular studies in Indian EOCRC patients have also found a decrease in *KRAS* mutations[99] and deregulation of

MAPK and *PI3K/AKT* pathways[100] compared with LOCRC patients. Whether EOSRC in Indian patients is molecularly distinct from Western or Caucasian patients, from whom most available CRC data were collected, remains unclear.

A recent study by Xu *et al*[43] compared germline mutations in a Western Caucasian EOCRC cohort to a Chinese EOCRC cohort and found the Chinese cohort had significantly fewer hereditary syndromes, with no germline *APC* mutations (mutated in 13% of the western cohort) or *BRCA1*, *SMAD4*, or *CHEK2* mutations, while these genes were mutated in 16% of western cohort patients under 50-year-old (330 Chinese and 430 Caucasian)[43]. Another recent molecular study in China examined clinical information for 947 EOCRC and 3521 LOCRC and found that EOCRCs were more likely to have a family history of cancer, higher TNM stage, and higher 3-year overall survival, but also a lower 3-year disease-free survival[101]. EOCRCs were also more likely to have defective MMR[101], though it is unclear whether this is a product of Lynch syndrome or sporadic mutations.

While data on CRC age-of-onset are available from many European and Asian countries, limited information on CRC epidemiology is available from countries in Africa[94]. New data identified an increased prevalence of CRC, with most African countries where data is available reporting an average age of CRC diagnosis between 43-year-old and 46-year-old[102]. One study compared EOCRC in Nigerians and African Americans (AA) and found that over 60% of Nigerian CRC patients were diagnosed before the age of 50-year-old, compared with 13.2% of AA[103]. The authors identified many differences between the two populations, where Nigerian EOCRCs were younger and had more rectal cancers[103]. Unfortunately, the demographic patterns of EOCRC in black individuals remain severely understudied[103].

In the United States, a clear racial disparity in CRC diagnosis and treatment exists, where the median age of CRC diagnosis is 68 for whites and 64 for blacks[1]. A study by Galadima *et al*[104] found that young AA had higher CRC incidence compared with young individuals of other races[104]. In addition, EOCRC rates in the United States are highest among Indigenous and black Americans[105]. Unfortunately, non-Hispanic blacks with EOCRC have a significantly worse 5-year survival than their white counterparts[106,107]. Previous studies have demonstrated ethnicity-specific differences in underlying CRC mutations[108], but few have focused on EOCRC, especially in people of African descent. One study did find a decrease in the prevalence of *APC* mutations and an increase in gene methylation in an AA EOCRC cohort compared to the mostly white CRC dataset provided by The Cancer Genome Atlas [109].

Overall, the incidence of CRC is increasing in young patients on a global scale, likely due to dietary and lifestyle changes across the world[94]. EOCRC may present differently and have different mutations in different populations around the world, likely owing to both lifestyle and genetic differences[43,97,103,108]. Therefore, it is crucial to increase our understanding of unique EOCRC drivers and to translate this knowledge to improve clinical outcomes for patients worldwide.

EOCRC biomarkers

The majority of EOCRCs are diagnosed between the ages of 40-49[110], leading to the American Cancer Society lowering the recommended CRC screening age from 50 to 45 in 2018[19]. However, several concerns remain on the cost/benefit analysis of this decision[111], indicating a crucial need for cost-effective early screening options.

While colonoscopy remains the gold standard of CRC screenings, blood and fecal tests are cheaper and less invasive options. Blood-based miRNA and DNA methylation biomarkers have been shown to accurately identify EOCRC[112]. There is currently one FDA-approved blood-based CRC screening test, Epi proColon®, which measures methylation of the gene *SEPT9* in cell-free DNA in serum. A recent study showed that methylation of *SEPT9* could accurately distinguish EOCRC patients from healthy controls, indicating that this test is effective for younger patients[112]. Another study suggested that the DNA repetitive elements *LINE-1*, *Sat2*, and *Alu* are hypermethylated in the white blood cells isolated from EOCRC patients, providing an additional potential methylation biomarker signature[62]. In addition to DNA methylation, miRNA expression is gaining popularity as a potential blood-based cancer biomarker. A recent study identified a miRNA signature of four miRNAs that could distinguish both EOCRC and LOCRC serum from healthy controls (Table 3)[75]. Serum expression of inflammatory genes has also been suggested to identify EOCRC patients, and one study found that the chemokine *CXCL12* has lower expression in younger compared to older patients[74].

Another minimally invasive screening option is a fecal test, such as Cologuard™, which measures methylation of the genes *BMP3* and *NDRG4* and assesses samples for the *KRAS* mutation. Cologuard also includes a fecal immunohistochemical test (FIT), which measures human globin, or blood, in the stool (Figure 2). Studies of whether these biomarkers can detect EOCRC are limited, though recent studies have found no significant difference in these markers within CRC tumors in younger and older patients[113-115]. Therefore, while Cologuard and FIT may be effective to detect EOCRC in fecal samples, additional studies are required before such recommendations can be made.

Gene expression biomarkers within tumors have also been suggested to serve as prognostic indicators. *miR-31-5p* was found to be uniquely overexpressed in sporadic EOCRC tumors *vs* normal samples, while it was not overexpressed in LOCRC. Moreover, the *miR-31-5p* target, *DMD*, was also shown to be decreased in EOCRC tumors, and this change in expression correlated with a worse

Table 3 Early-onset colorectal cancer biomarkers

Name	Type	Description
mSEPT9	Methylation, DNA	Blood-based biomarker used in Epi proColon® or both EOCRC and LOCRC[112]
miR-193a-5p, miR-210, miR-513a-5p, miR-628-3p	miRNAs	miRNA in serum, panel works for both EOCRC and LOCRC[75]
Sat2, LINE-1, Alu	Methylation, DNA	DNA repetitive elements with increased methylation in EOCRC in white blood cells[62]
miR-31-5p, DMD	miRNA, mRNA	Transcripts uniquely overexpressed in sporadic EOCRC tumor *vs* normal and not in LOCRC. *miR-3105p* targets *DMD* and it's downregulated in EOCRC[116]
MYC	mRNA	Transcription factor with increased tumor expression in EOCRCs may subset patients into distinct groups[50,69]

EOCRC: Early-onset colorectal cancer; LOCRC: Later-onset colorectal cancer; miRNA: microRNAs.

prognosis (Table 3)[116]. In addition to genetic, transcriptomic, and proteomic alterations, biomarkers of the gut microbiome have also been suggested to identify EOCRC, as EOCRC has been shown to have a distinct microbiome compared with LOCRC[117]. Microbiota studies in CRC and EOCRC are outside the scope of this review but are nicely summarized by Abdullah *et al*[118]. Overall, a limited number of studies have shown that common CRC screening options may apply to EOCRC as well. miRNA and DNA methylation biomarkers have been proposed to help identify EOCRC (Table 3), however additional studies with larger sample sizes and clinical validations are required.

Currently, clinical practice guidelines do not differentiate the treatment of EOCRC *vs* LOCRC[119]. However, CRC treatment is dependent on tumor mutational profiling, and thus, the lower frequency of *BRAF*[34,38,57] and *KRAS*[99,120] mutations in EOCRC means the mutation-specific treatments will be less commonly used in EOCRC patients. The efficacy of these drugs in EOCRC has not been directly studied but the mechanism is likely very similar to LOCRC.

CONCLUSION

Several clinical features have been associated with EOCRC. Approximately 75% of sporadic cases occur in the 40–year-old to 49-year-old age group, with 55%-80% of EOCRCs occurring in the distal colon or rectum[121,122]. The increasing rate of EOCRC has been predominated by an increasing rate of distal colon and rectal cancer, with individuals born circa 1990 having double and quadruple the risk of colon and rectal cancer, respectively, compared to those born circa 1950[123]. While many strides have been made to understand alterations at the DNA, RNA, and protein levels that contribute to EOCRC, questions remain on how EOCRC patients should be treated compared with their older counterparts. Currently, young patients are more likely to be treated, or overtreated, with systemic chemotherapy, but have similar clinical outcomes compared with older patients[10,104,124].

As the number of EOCRC cases continues to rise globally, there is a critical need to optimize cancer treatment strategies, as well as to further develop non-invasive screening options to identify people at risk for EOCRC. Currently, scientific studies are limited by low sampling size, especially in non-white patients. Researchers are addressing this limitation by continuing to grow biobanks with younger and non-diseased samples. Furthermore, machine learning approaches have been suggested to increase the statistical power of limited sample sizes[125], which could be applied to identify EOCRC risk genes or genetic loci in under-represented minorities. Another limitation is the lack of a clear model system to test hypotheses on EOCRC. While models for EOCRC exist, as *APC*^{min} mice develop CRC at a young age, and HCT-116 cells are from a young patient[126], these systems fail to recapitulate the diversity in EOCRC subtypes that are observed in patient samples. The development of stable cell lines from CRC samples generally requires transformation, altering the cellular profiles, and limiting normal controls. Organoid models are gaining popularity due to their ability to recapitulate the colonic crypt structure from both normal and tumor cells[33,127]. Future work will tease out the molecular mechanisms unique to EOCRC with growing biobanks and organoids as well as other innovative model systems.

FOOTNOTES

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MINIREVIEWS

Anticancer potential of *Ferula assa-foetida* and its constituents, a powerful plant for cancer therapy

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Abstract

Cancer is one of the main challenges of the health system around the world. This disease is increasing in developing countries and imposes heavy costs on patients and governments. On the other hand, despite various drugs, the death rate among cancer patients is still high and the current treatments have many harmful effects. In the traditional medicine of different countries, there are many medicinal plants that can be effective in the treatment of cancer. Ferula plants are traditionally used as spices and food or for medicinal purposes. *Ferula assa-foetida* is one of the famous plants of this genus, which has been used for the treatment of various diseases since ancient times. Among the main compounds of this plant, we can mention monoterpenes, sulfide compounds and polyphenols, which can show different therapeutic effects. This article has been compiled with the aim of collecting evidence and articles related to the anti-cancer effects of extracts, derived compounds, essential oils and nanoparticles containing *Ferula assa-foetida*. This review article was prepared by searching the terms *Ferula assa-foetida* and cancer, and relevant information was collected through searching electronic databases such as ISI Web of Knowledge, PubMed, and Google Scholar. Fortunately, the results of this review showed that relatively comprehensive studies have been conducted in this field and shown that *Ferula assa-foetida* can be very promising in the treatment of cancer.

Key Words: *Ferula assa-foetida*; Anticancer; Essential oil; Isolated components; Nano particle; Extract

Core Tip: Finding new anti-cancer compounds is an important necessity in the treatment or prevention of this disease. *Ferula assa-foetida* has useful compounds for the prevention and treatment of cancer, which can be used in making new compounds. These compounds include sulphide compounds, flavonoids and terpene coumarins, which with new methods such as making emulsions and nanoparticles from these compounds can be of great help in reducing the costs of cancer patients and their life expectancy.

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INTRODUCTION

Today, one of the main problems of the health community is cancer, which is currently known as the second leading cause of death in the world. The most common cancers are breast and lung cancer worldwide, accounting for 12.5% and 12.2% of all newly diagnosed cases, respectively[1]. Common treatments include radiotherapy and chemotherapy that stop the cell cycle through apoptosis or non-apoptosis mechanisms such as necrosis[2]. These therapies have a variety of side effects, including damage to healthy cells. Medicinal plants have therapeutic value due to their biologically active compounds such as terpenes, coumarins, phenolic and alkaloids[3]. These natural compounds have shown promising insight into the treatment and prevention of cancer by restricting the division of tumor cells or inducing apoptosis with the advantage to reduce side effects[4]. The genus Ferula includes 170 different species that are distributed all over the world and this genus belongs to the Apiaceae (Umbelliferae) family[5]. *Ferula assa-foetida*, one of the famous species of Ferula that is used in Iranian traditional medicine for the treatment of digestive diseases, nervous problems and some reproductive system disorders such as decreased libido[6]. Asafoetida or Anghouzeh (Traditional name in Persian), is an oleo gum resin which obtained from the root of *Ferula assa-foetida* and traditionally used as anthelmintic, anticonvulsant, sexual aphrodisiac and analgesic agent[7]. New scientific reports have shown that asafoetida has antifungal[8], antidiabetic[9], antiinflammatory[10], antimutagenic[11] antidementia[12], anticonvulsant[13], antiviral[14], anti-cancer[15] and relaxant[16] activities and also has preventive effect against cuprizone induced demyelination[17]. There is not enough information available about the dosage and toxicity of asafoetida, but it is recommended not to consume more than 0.2 g per day[18], and it has also been shown that long-term and high-dose administration (200 mg and above) causes liver damage[19]. The main compounds that have been identified in the *Ferula assa-foetida* include glycoside compounds, various terpenoid, coumarin derivatives, and sulfide compounds[20,21] which have been shown to have anti-cancer potential (Figures 1 and 2).

Some compounds isolated from *Ferula assa-foetida* have also been shown to have various pharmacological properties. For example, Ferulic acid is one of these compounds that has antioxidant and neuroprotective properties[22]. Umbelliferon is a coumarin compound that has antioxidant and antidiabetic as well as antitumor effects[23]. In recent years, many studies have been conducted on the anti-cancer effects of Ferula. The members of this genus have shown high anti-cancer potential, which can provide a good basis for finding new anti-cancer agents. Our focus on published studies on the impact of different extracts and compounds isolated from *Ferula assa-foetida* as anticancer agents. Due to the increase in cancer patients and significant findings on the anticancer effects of *Ferula assa-foetida*, this article is designed for help to researchers finding new anticancer compounds.

METHOD

This review article was prepared by searching the terms of *Ferula assa-foetida* and cancer. Information about *Ferula assa-foetida* and its anticancer effect was collected on electronic databases including ISI Web of Knowledge, Medline/PubMed, ScienceDirect, Embase, Scopus, Biological Abstract, Chemical Abstract and Google Scholar. To make the research easier to understand, the article is divided into different sections, including the anti-cancer effects of nanoparticles containing *Ferula assa-foetida*, essential oils, extracts, isolated compounds from *Ferula assa-foetida*, and preclinical and experimental studies (Table 1).

Table 1 An overview of anticancer effect of different parts of *Ferula assa-foetida*

	Type/name	Cell line	Effects	Ref.
Nano particle	Silver nanoparticles and asafoetida ethanol extracts	L6 cancer cell line	IC50 was calculated 1 μg/mL	Subramaniam *et al* [25], 2021
	Nano emulsion containing *Ferula assa-foetida* seed essential oil	MCF7 and A2058 cell line	Increased BAX and decreased BCL2 expression. IC50 = 64 μg/mL for MCF7 and 201 μg/mL for A2058. Also, decreased VEGF at 32 μg/mL and VEGFR at 128 μg/mL	Azani *et al*[26], 2021
	Lipid nanoparticles containing *Ferula assa foetida* seed oil	NT-2 human cancer stem cells	IC50 = 115.4 μg/mL and the number of blood vessels reduced at 250, 500, and 1000 μg/mL	Sadat Khadem *et al* [27], 2021
	Silver anoparticles (AgNPs) with aqueous extract of asafoetida	MCF-7	IC50 was calculated 2 μg/mL	Devanesan *et al*[28], 2020
	Zinc nanoparticles containing *Ferula assa-foetida* extract	MCF7, MDA-MB231 and HT-29	IC50 was 23, 41.26 and 143 μg/mL after 72 h	Boskabadi *et al*[29], 2020
	Ferula assa foetida essential oil on PLGA nanoparticles	HepG2 and A2780	Inhibited HepG2 and A2780 with an IC50 of 57 μg/mL and 106.7 respectively. Reduction of vascular parametric factors at 125 μg/mL	Mokhtareeizadeh *et al*[30], 2021
Essential oil	(-)-E-2-butylpropenyl disulfide, (-)-Z-2-butylpropenyl disulfide, (-)-1-(methylthio) propyl (E)-1 -Propenyl disulfide, and (-)-1-(methylthio) propyl (Z)-1-propenyl disulfide	SKOV3 (ovary) and A549 (lung) cancer cell lines	Trisulfide showed better activity against A549 and SKOV3 cell lines compared to disulfides	Yatham *et al*[31], 2021
	Seed of *Ferula assa foetida* essential oil	AGS gastric cancer cells	Inhibitory effect on AGS gastric cancer cells was near 100% at 10 μl/mL after 72 h incubation	Bagheri *et al*[32], 2020
	Asafoetida essential oil	HepG2 and SK-Hep1	IC50 for HepG2 and SK-Hep1 was 7.21 μg/mL and 8.0 μg/mL respectively	Verma *et al*[33], 2019
	Essential oils asafoetida and	T98G and HCT116	IC50 value for HCT116 was 5.96 μg/mL and for T98G was 4.49 μg/mL	Pavela *et al*[34], 2020
	Essential oil of asafoetida	MCF7 cells	Decreased the viability of MCF7 cells in a time and concentration-dependent manner	Bagheri *et al*[35], 2020
Isolated components	Ferulic acid	MDA-MB-231	Combination with 25 μM of thymoquinone and 250 μM of ferulic acid, decrease proliferation of MDA-MB-231 cells	Al-Mutairi *et al*[38], 2021
	Ferulic acid	MDA-MB-231	Increased caspase 3 and reduced the proliferation of cancer cells about 40% at 100 μM. 100 mg/kg significantly reduced tumor volume, weight and growth in mice	Zhang *et al*[39], 2016
	Ferulic acid	4T1 cells	Reduced the growth of cancer cells at 500 μg/mL	Bagheri *et al*[40], 2017
	Galbanic acid	MDA-MB-231 and MCF-7 cells	IC50 was 48.7 and 56.6 μg/mL, respectively. Up-regulation of Bax and caspase-3 and down-regulation of bcl2	Sajjadi *et al*[42], 2019
	Galbanic acid	H460, A549, PC-9 and HCC827	IC50 calculated 100 μM on H460 cell line. Bax and caspase 9 increased and Bcl-2, Bcl-xL and myeloid cell leukemia 1 (Mcl-1) decreased in H460 cells	Oh *et al*[43], 2015
	Galbanic acid	AR+ PCa cells and AR- PCa cells	Suppresses the growth of AR (+) PCa cells. Inhibited cyclin/CDK4/6 pathway, specially cyclin D1	Zhang *et al*[44], 2012
	Farnesiferol C	HUVEC and mouse Lewis lung cancer cells	10-40 μmol/L inhibited VEGF. Reduced the growth of mouse Lewis lung cancer by 60%	Lee *et al*[45], 2010
	Sesquiterpene coumarins	PC-3 and MCF-7	Gummosin showed highest cytotoxic activity. Also showed an IC50 values at 30 and 32.1 μg/mL against PC-3 and MCF-7 cell lines respectively	Iranshahy *et al*[48], 2019
	Farnesiferol C	MCF-7	Decrease cell viability after 24, 48 and 72 h. (IC50 43, 20 and 14 μM, respectively), and stopped the cell cycle in G0/G1 phase and induced apoptosis in MCF-7 cells	Hasanzadeh *et al* [46], 2017

AGS: Aerobic granular sludge; BCL2: B-cell lymphoma 2; EMT: Epithelial-mesenchymal transition; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor.

ANTICANCER EFFECT OF NANOPARTICLES CONTAINING *FERULA ASSA-FOETIDA*

Encapsulation of essential oils, extracts and plant derivatives can overcome their therapeutic limitations and lead to better stability, increased bioavailability and better efficacy[24]. The use of nanoparticles in cancer treatment is a new method that can be used to target treatment. *Ferula assa-foetida* has various biological compounds that make it a suitable candidate for use in cancer treatment. Various studies have been conducted on the effect of different derivatives and extracts of this plant on different cell lines of cancer cells and generally positive results have been obtained. For example, use of silver nanoparticles and ethanol extract of asafoetida caused a decrease in the survival rate of L6 cancer cells, and the IC_{50} value was calculated as 1 μg/mL[25]. Some studies have shown that nanoemulsion containing *Ferula assa-foetida* essential oil can cause apoptosis by increasing BAX expression and decreasing BCL-2 in MCF7 cancer cells. The lethality of this nanoparticle has been calculated based on IC_{50} equal to 64 μg/mL for MCF7 and 201 μg/mL for A2058. Also, a significant decrease in the expression of vascular endothelial growth factor (VEGF) at 32 μg/mL and vascular endothelial growth factor receptor (VEGFR) at 128 μg/mL was observed in MCF-7 cells treated with nanoemulsion. This nanoparticle was able to significantly reduce tumor indices in the murine model of induced breast cancer at a concentration of 100 mg/kg[26]. Lipid nanoparticles containing *Ferula assa-foetida* seed oil on NT-2 human cancer stem cells had an IC_{50} equal to 115.4 μg/mL. The morphometric results of blood vessels treated with these nanoparticles showed that the number of blood vessels was significantly reduced in concentrations of 250, 500 and 1000 μg/mL in a dose-dependent manner. Also, these nanoparticles increased the expression of TNF-α, P21, and Cas3[27]. Synthesis of silver nanoparticles (AgNPs) with aqueous extract of asafoetida on MCF-7 cells caused cell death in a dose-dependent manner and its IC_{50} was calculated as 2 μg/mL[28]. By making zinc nanoparticles containing *Ferula assa-foetida* extract and investigating its effects on MCF7, MDA-MB231 and HT-29 cell lines, Boskabadi *et al*[29] showed that this nanoparticle can significantly reduce the growth of cancer cells. The calculated IC_{50} was equal to 23, 41.26 and 143 μg/mL after 72 h, respectively. In addition, the results showed that the nanoparticle has apoptotic properties and antioxidant activity with an IC_{50} equal to 500 mg/mL. Expression of Bax and Bcl2 significantly up and down regulated respectively. Mokhtareeizadeh *et al*[30] founded that nanoparticles containing *Ferula assa-foetida* essential oil can inhibit the growth of HepG2 and A2780 cells with IC_{50} of 57 and 106.7 μg/mL respectively. These nanoparticles caused a significant decrease in angiogenesis in fertilized eggs at a dose of 125 μg/mL. Also it induced apoptosis and death of cancer tissue cells by regulating Caspase3 and 9, TNF-α, P53 and P21 in nude mice with breast cancer.

ANTICANCER EFFECT OF ESSENTIAL OIL OF *FERULA ASSA-FOETIDA*

The main part used by *Ferula assa-foetida* is an oleo gum resin, which is obtained by shaving its root. This oleo gum resin contains many different compounds, the anti-cancer effects of some of these compounds have been investigated. The volatile part of oleo gum resin or its essential oil contains generally sulfur compounds that have a pungent and unpleasant smell. Some studies have shown that essential oil has strong anti-cancer effects. For example, Yatham *et al*[31] found four main compounds in asafoetida essential oil, including (-)-E-2-butylpropenyl disulfide, (-)-Z-2-butylpropenyl disulfide, (-)-1-(methylthio) propyl (E)-1 -Propenyl disulfide, and (-)-1-(methylthio) propyl (Z)-1-propenyl disulfide were identified and investigated their potential to inhibit the growth of cancer cell lines SKOV3 (ovary) and A549 (lung). Meanwhile, trisulfide showed better activity against A549 and SKOV3 cell lines compared to disulfides. The analysis of *Ferula assa-foetida* seed essential oil showed that it contains compounds such as E-1-propenyl sec-butyl disulfide (13.13%) Z-1-propenyl sec-butyl disulfide (11.34%). This essential oil exerted its inhibitory effect on aerobic granular sludge gastric cancer cells near 100% in 10μl/mL in 72 h after incubation[32]. The anti-proliferative and anti-apoptotic effects of asafoetida essential oil on liver cancer cell lines (HepG2 and SK-Hep1) as well as the expression of NFKB1, TGFB1, TNF, and caspase3 genes showed that the IC_{50} of the oil for HepG2 and SK-Hep1 was 7.21 μg/mL and 8.0 μg/mL respectively. After EO treatment, the genes involved in metastasis and proliferation decreased and the genes involved in apoptosis showed a significant increase (casp3 and TNF). Analysis of the essential oil by GC showed the presence of 1, 2-dithiolane in the amount of 87.4%[33]. Pavela *et al*[34] evaluated the essential oils asafoetida and *Ferula gummosa* on T98G (human glioblastoma multiforme cell line), HCT116 (human colon cancer cell line). *Ferula assa-foetida* essential oil was more active on HCT116 with IC_{50} value of 5.96 μg/mL and *Ferula gummosa* essential oil showed more activity on T98G with IC_{50} value of 4.49 μg/mL. Essential oil of asafoetida (EOA) exposed MCF7 cells to different concentrations of EOA (2, 4, 6, 8, and 10 μl/mL) at 24, 48 and 72 h showed that EOA significantly decreased the viability of MCF7 cells in a time and concentration-dependent manner. The

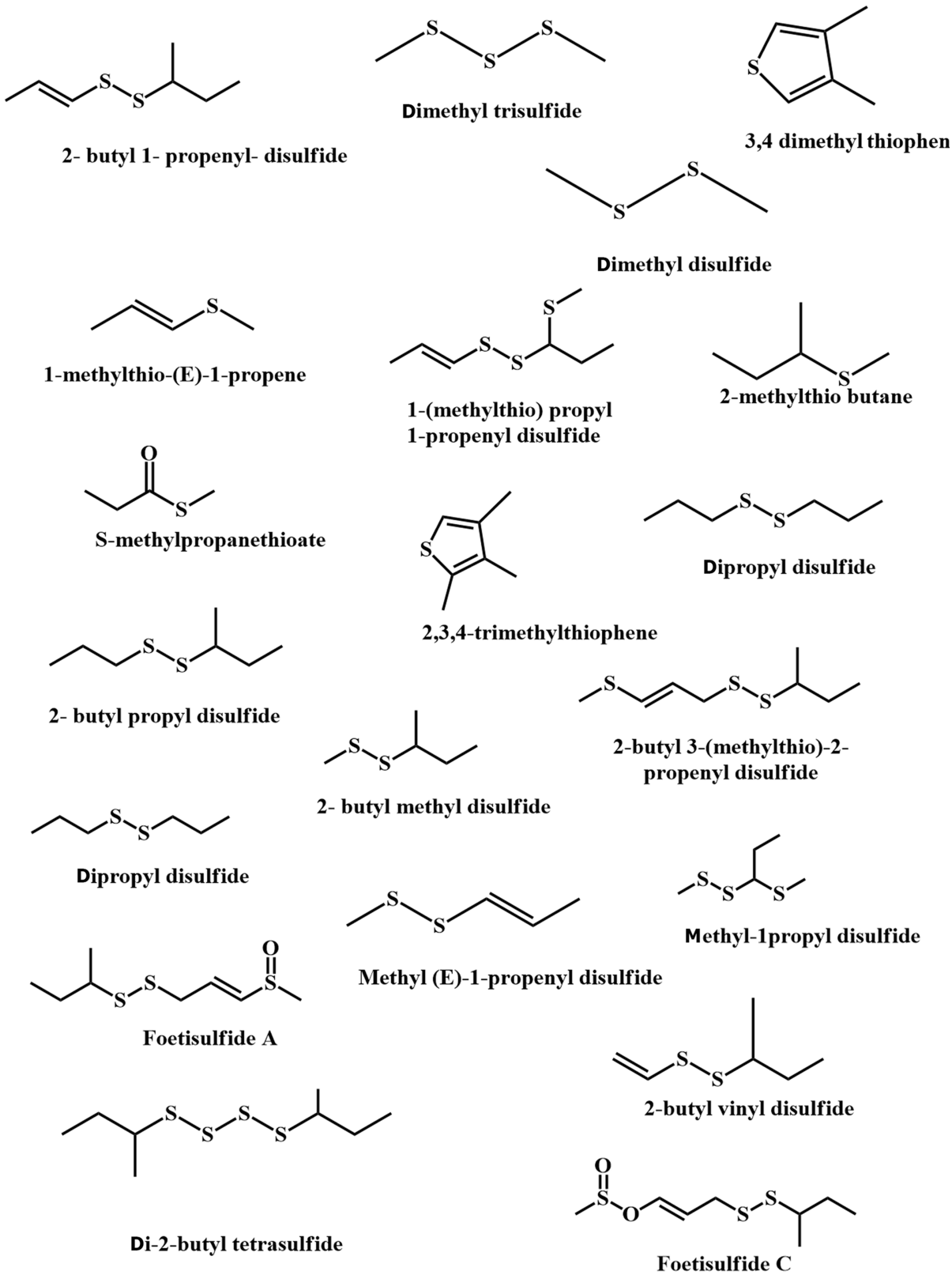

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Figure 1 Chemical structure of some sulfide compounds derived from Ferula assa-foetida.

major constituents identified in EOA were E1propenyl secbutyl disulfide (36.15) and Z1propeny secbutyl disulfide (27.93%)[35]

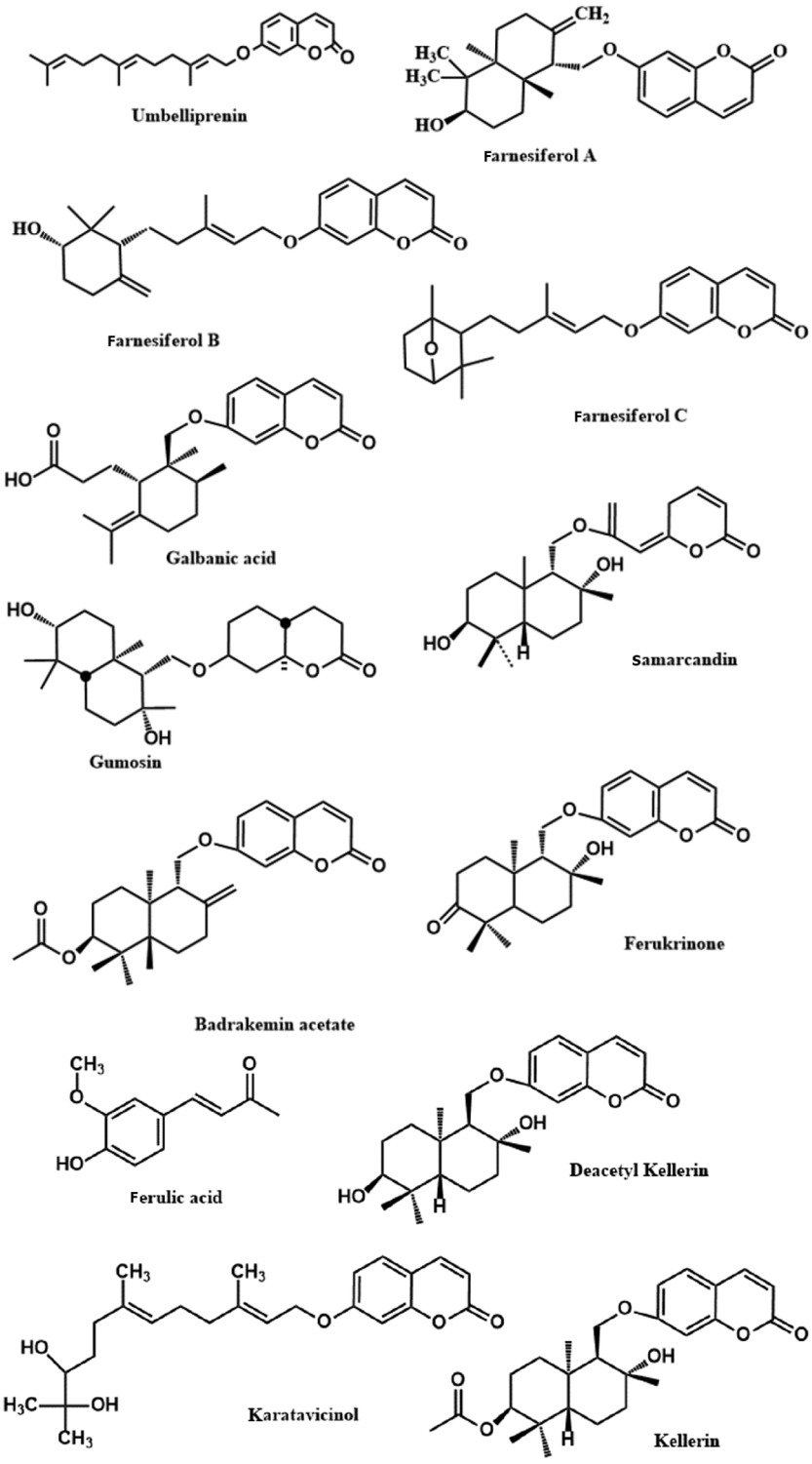

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Figure 2 Chemical structure of isolated constituents from *Ferula assa-foetida* showed anticancer effect.

ANTICANCER EFFECT OF ISOLATED CONSTITUENTS FROM *FERULA ASSA-FOETIDA*

Several compounds are derived from *Ferula assa-foetida*, which include coumarins, sesquiterpene coumarins, flavonoids and phenolic constituents that have shown a number of pharmacological effects, including antibacterial, antifungal, cytotoxic, antioxidant and hormonal activities, as well as anticancer effects[36]. Ferulic acid is one of the phenolic compounds in asafoetida, which has various therapeutic effects[37]. Al-Mutairi *et al*[38] have shown that when ineffective doses of ferulic acid were used with ineffective doses of thymoquinone, it was able to significantly reduce the death of MDA-MB- cells after 48 h. In another study, ferulic acid increased caspase 3 activity in the breast cancer cell line MDA-MB-231 and reduced the proliferation of the cancer cell line about 40% after 72 h at a concentration of 100

μM. Also, the anti-tumor potential of ferulic acid in a xenograft mouse model with MDA-MB-231 at a concentration of 100 mg/kg body weight could reduce tumor volume, weight and growth[39]. Bagheri *et al*[40], showed that ferulic acid significantly reduced the growth of 4T1 mouse breast cancer cells at a dose of 500 μg/mL. Galbanic acid is a terpenes lactone derived from the gum of *Ferula assa-foetida*, which has also been identified in several other species of Ferula[41]. Treatment of MDA-MB-231 and MCF-7 cells with galbanic acid showed that this compound leads to the inhibition of proliferation and induction of apoptosis with IC_{50} of 48.7 and 56.6 μg/mL, respectively. Also, galbanic acid stimulated apoptosis through the up-regulation of Bax and caspase-3 and the down-regulation of bcl2 and increased the expression of superoxide dismutase, catalase and glutathione peroxidase genes[42]. In confirmation of these results, in another study, the potential of galbanic acid in inhibiting four types of non- small lung cancer cells H460 and A549, PC-9 and HCC827 were proven after 24 h. Meanwhile, H460 cell line has the highest sensitivity to galbanic acid and showed an IC_{50} of about 100 μM. It was also found that the expression levels of Bax and caspase 9 increased and Bcl-2, Bcl-xL and myeloid cell leukemia 1 (Mcl-1) decreased and cleaved poly (ADP-ribose) polymerase (PARP) in H460 cells[43]. Androgen receptor (AR) signaling is crucial for the initiation and progression of prostate cancer (PCa). In a study, it was found that galbanic acid preferentially suppresses the growth of AR (+) PCa cells compared to AR (-) PCa cells. Galbanic acid induces apoptosis through G1 arrest associated with inhibition of cyclin/CDK4/6 pathway, especially cyclin D1[44]. The anti-angiogenic activities of farnesiferol C (FC) in human umbilical vein endothelial cells showed that exposure to a concentration range of 10-40 μmol/L FC inhibited VEGF, migration, invasion cells and decrease the expression of matrix metalloproteinase 2. Furthermore, FC inhibited the angiogenesis of mouse aorta treated with VEGF in an experimental model. FC reduced the growth of mouse Lewis lung cancer by 60% and caused rapid inhibition of VEGFR1 autophosphorylation caused by VEGF without affecting VEGFR2. However, FC inhibited the phosphorylation of most VEGFR2 downstream kinases such as focal adhesion kinase, Src, extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase, and c-jun-NH2-kinase without affecting AKT[45]. Sesquiterpene coumarins are a group of compounds found in the genus Ferula that have shown various therapeutic effects such as anticancer effects[21]. Farnesiferol C obtained from the chloroform extract of *Ferula assa-foetida*, on MCF-7 cells, led to a decrease in cell viability after 24, 48 and 72h. (IC50 43, 20 and 14 μM, respectively). Farnesiferol C stopped the cell cycle in G0/G1 phase and induced apoptosis in MCF-7 cells. This compound increased cellular SOD, CAT MDA activities in 24 and 48 h and reduced activity of SOD and CAT and increased MDA level after 72 h exposure. It demonstrated that reactive oxygen species level increased 5.92%, 13.53% and 14.43% after 24, 48 and 72 h exposure, respectively[46]. Treatment of K562, KBM5, U937 and HL-60 cancer cells with farnesiferol C showed that this substance has an IC_{50} = 10 μM on K562 cells and 20μM on KBM5 cells and showed a significant effect only on these two types of cells. Also, cleaved PARP and caspase 3 and 9 decreased the expression of Bcl2 and stopped cells in G1, and farnesiferol C decreased the expression of Cyclin D1, Cyclin E, Cyclin B1 and histone deacetylase 1 and 2 in K562 and KBM52 cells[47]. Investigation on anticancer potential of ten sesquiterpene coumarins include farnesiferol A, farnesiferol B, farnesiferol C, gummosin, samarkandin, umbelliprenin, badrakemine acetate, ferukrinone, kellerin and deacetyl kellerin derived from asafoetida showed that gummosin has highest cytotoxic activity among these sesquiterpene coumarins. It showed an IC_{50} values of 30 and 32.1 μg/mL against PC-3 and MCF-7 cell lines respectively[48]. Umbelliprenin is a prenylated coumarin compound found in Ferula species, also isolated from *Ferula assa-foetida*. This structure has various pharmacological effects such as cytotoxic activities and induction of apoptosis[49]. Using the umbelliprenin isolated from *Ferula assa-foetida* on Jurkat T-CLL and Raji B-CLL cell lines showed that umbelliprenin induced apoptosis in a dose- and time-dependent manner (IC50, 16 h = 75 μM and 48 h = 25 μM respectively)[50]. Farnesylation of the activated oncogenic ras product by Farnesyltransferase (FTase) is a critical step for its oncogenic function. Isolation of galbanic acid, karatavicinol, umbelliprenin, farnesiferol B, farnesiferol C from *Ferula assa-foetida* to inhibit FTase showed that galbanic acid has the highest enzyme inhibition potential and IC_{50} was calculated as 2.5 μM. In addition, the calculated IC_{50} value in reducing the proliferation of oncogenic ras-transformed NIH3T3/Hras-F cells by galbanic acid was 16.2 μM compared to the control group[51].

DIFFERENT EXTRACTIONS OF *FERULA ASSA-FOETIDA* ON CANCER

Ferula assa-foetida ethanolic extract showed a significant effect on PC12 and MCF7 cells in reducing cell survival. The amount of IC_{50}s for 24, 48 and 72 h for MCF7 was 1.30, 1.284, 0.753 μM, respectively. Also, IC_{50}s for PC12 category at 24, 48 and 72 h were calculated as 2.84, 0.8 and 0.4 μM, respectively[52]. The petroleum benzene, chloroform and methanol extract of asafoetida on MCF7 HepG2, A549, HT-29 and MDBK showed that the methanol fraction has an IC_{50} of more than 100 μg/mL. Petroleum and chloroform extracts showed IC_{50} values less than 52 μg/mL in four cell lines. Chloroform fraction showed IC_{50} equal to 61.42 μg/mL in MCF7. The petroleum afraction showed an IC_{50} of 45.73 μg/mL in MCF7[53]. The hydroalcoholic extract of *Ferula assa-foetida* significantly reduce the mRNA expression level of epithelial-mesenchymal transition markers (vimentin, Snail1, Zeb1) and the anti-apoptotic

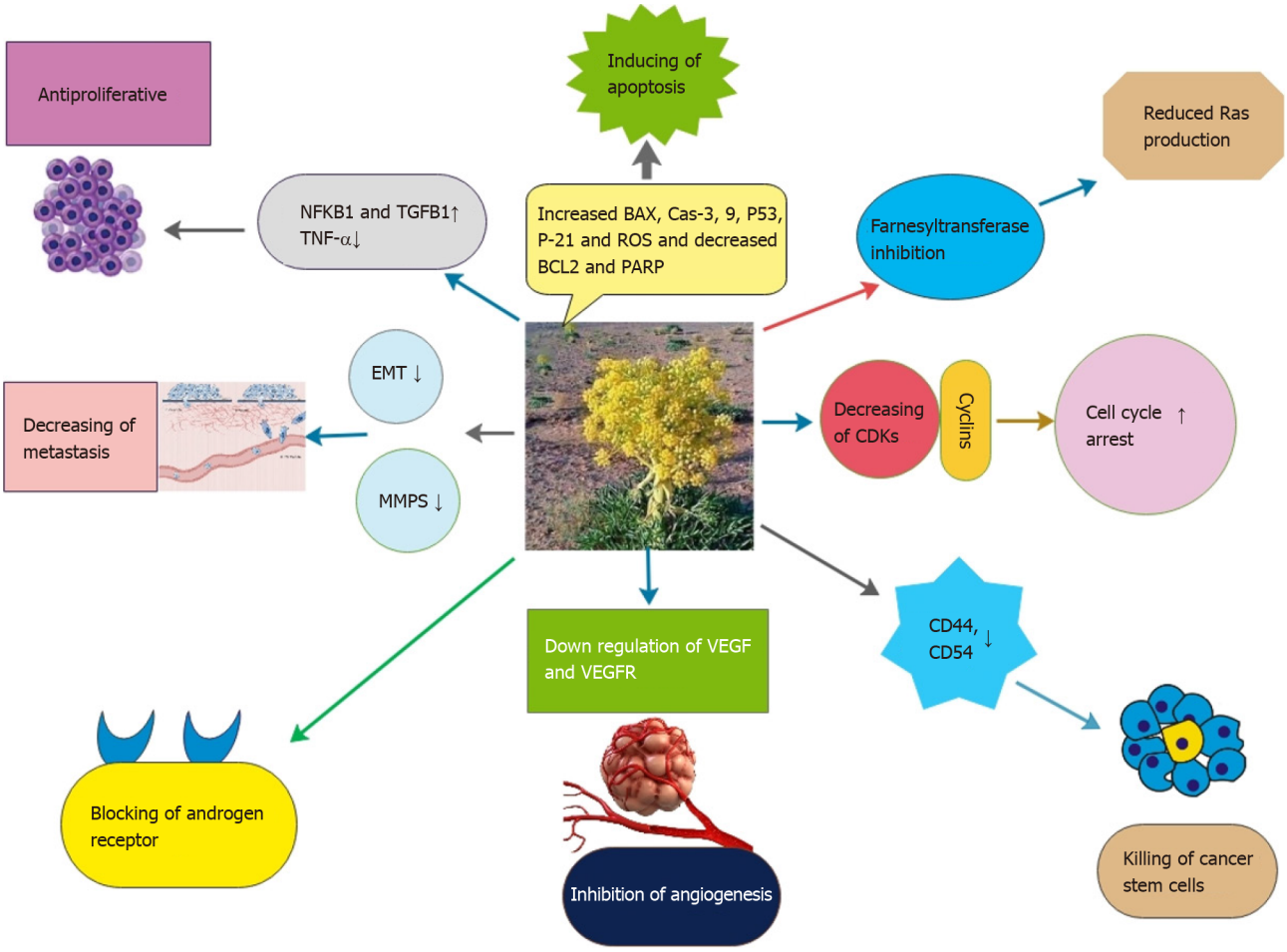

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Figure 3 Investigated mechanisms by which *Ferula assa-foetida* exerts its anticancer effects. BCL2: B-cell lymphoma 2; CDKs: Cyclin-dependent kinases; EMT: Epithelial-mesenchymal transition; MMPS: Matrix metalloproteinases; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; ROS: Reactive oxygen species.

marker Bcl-2, as well as the expression of stem cell marker CD44 and CD54[54]. Ethanol extracts of *Ferula assa-foetida* and a number of its components (ferulic acid, vanillic acid, quercetin, ellagic acid, and p-coumaric acid) had cytotoxic effects on MCF-7 or MDA-MB-231 human breast cancer cells and 4T1 mouse cell line. Also, THP-1 peripheral blood monocytic leukemia cells can be polarized to M1 inflammatory phenotype by treatment with the extract and its components. Furthermore, this THP-1-dependent polarization of macrophages demonstrated an enhanced ability to damage MCF-7 or MDA-MB-231 cell monolayers in co-culture experiments. Therefore, treatment with *Ferula assa-foetida* extract can also indirectly cause the death of cancer cells through the activation of immune cells[55]. The cytotoxic effects of the ethanolic extract of Ferula assa-foetida resin on HepG2 cell line in concentrations (10, 50, 100, 200 μg/mL) showed that this extract in doses of 50, 100 and 200 μg/mL decreased the viability of HepG2 cells but in doses of 100 and 200, it also changes the shape of normal L929 cells. Therefore, only a dose of 50 μg/mL can be considered as an effective and non-toxic dose[56]. The investigation of methanolic and ethanolic extract of *Ferula assa-foetida* resin on osteosarcoma cell line showed that different concentrations of the extract in 24 and 48 h can reduce the survival of cancer cells. The highest effect rate corresponding to the concentration of 20 mg in 48 h for ethanolic and methanolic extract was calculated as 29.5 and 35.2, respectively. Also, the results showed that the ethanolic extract has a greater effect on the death of cancer cells[57].

ANIMAL EVIDENCES FROM ANTI-TUMOR EFFECT OF *FERULA ASSA FOETIDA*

Although animal evidence for the anticancer effect of *Ferula assa-foetida* is not much, several limited studies have shown that this plant has good anticancer potential. In a study, it was found that the use of 100 mg/kg asafoetida for 21 d against breast cancer caused by 4T1 cells in BALB/c mice can reduce tumor weight and tumor volume and increase the weight of treated mice. Also, asafoetida reduced lung, liver and kidney metastasis respectively. Asafoetida showed significant inhibitory activity against

lipoxygenase as well as antioxidant activity[15]. The use of food containing asafoetida (1.25 and 2.5%) showed that asafoetida significantly restored the level of the antioxidant system MNU (N-methyl-N-nitrosourea) induced mammary carcinogenesis in Sprague-Dawley rats. Furthermore, only in the MNU-control group, all animals had tumors with an average of 5.45 tumors per mouse (tumor burden) at the end of 18 wk, but the tumor burden in treated groups (1.25% and 2.5%) with asafoetida decreased to 3.6 and 2.3 tumor/mouse, respectively. The tumor volume in treated groups also decreased to 1.9cc (40%) and 1.3cc (59%), respectively, compared to 3.2cc in control group[58]. The use of different doses of asafoetida (5, 10 and 20 mg/100 g body weight) on dimethylhydrazine-induced colon cancer in rats showed that body weight, tumor frequency, tumor incidence, tumor size, total serum sialic acid as well as the tissue structure of the colon improved in all groups treated with asafoetida and these effects was better at dose of 10 mg/ 100 g body weight than other doses[59].

ANTICANCER MECHANISMS

The results of this study show that extracts and compounds isolated from Ferula asafoetida can cause the death of cancer cells in different ways. These mechanisms are briefly shown in Figure 3. As can be seen from this diagram, by reducing angiogenesis, increasing apoptosis, inhibiting metastasis, affecting the oxidative system of cancer cells and disrupting the cycle of cancer cells, *Ferula assa-foetida* causes damage and death of these cells.

CONCLUSION

Cancer is one of the serious problems of human society, especially in developing countries. The costs of treating the disease are very high and the death rate caused by it is worrying. The healthcare system and the research community should find effective and low-cost treatment methods as soon as possible, especially for poor communities. Finding anti-cancer compounds of natural origin is one of these solutions. It is very encouraging to see the results of the anti-cancer effects of *Ferula assa-foetida*. These results show that asafoetida can be considered as a medicinal plant in cancer treatment. Many of the effective compounds found in plant gum have anti-cancer effects, which can be inspired by these compounds to create new drugs. The use of asafoetida as a seasoning in foods can also be effective in the follow-up of cancer. By taking advantage of new methods such as nanotechnology and biotechnology, we can imagine a better perspective in using this plant and its derivatives as an anti-cancer agent.

FOOTNOTES

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ORIGINAL ARTICLE

Observational Study

Temporal pattern of humoral immune response in mild cases of COVID-19

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Abstract

BACKGROUND

Understanding the humoral response pattern of coronavirus disease 2019 (COVID-19) is one of the essential factors to better characterize the immune memory of patients, which allows understanding the temporality of reinfection, provides answers about the efficacy and durability of protection against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and consequently helps in global public health and vaccination strategy. Among the patients who became infected with SARS-CoV-2, the majority who did not progress to death were those who developed the mild COVID-19, so understanding the pattern and temporality of the antibody response of these patients is certainly relevant.

AIM

To investigate the temporal pattern of humoral response of specific immunoglobulin G (IgG) in mild cases of COVID-19.

METHODS

Blood samples from 191 COVID-19 real-time reverse transcriptase-polymerase chain reaction (RT-qPCR)-positive volunteers from the municipality of Toledo/Paraná/Brazil, underwent two distinct serological tests, enzyme-linked immunosorbent assay, and detection of anti-nucleocapsid IgG. Blood samples and clinicoepidemiological data of the volunteers were collected between November 2020 and February 2021. All assays were performed in duplicate and the manufac-

turers' recommendations were strictly followed. The data were statistically analyzed using multiple logistic regression; the variables were selected by applying the $P < 0.05$ criterion.

RESULTS

Serological tests to detect specific IgG were performed on serum samples from volunteers who were diagnosed as being positive by RT-qPCR for COVID-19 or had disease onset in the time interval from less than 1 mo to 7 mo. The time periods when the highest number of participants with detectable IgG was observed were 1, 2 and 3 mo. It was observed that 9.42% of participants no longer had detectable IgG antibodies 1 mo only after being infected with SARS-CoV-2 and 1.57% were also IgG negative at less than 1 mo. At 5 mo, 3.14% of volunteers were IgG negative, and at 6 or 7 mo, 1 volunteer (0.52%) had no detectable IgG. During the period between diagnosis by RT-qPCR/symptoms onset and the date of collection for the study, no statistical significance was observed for any association analyzed. Moreover, considering the age category between 31 and 59 years as the exposed group, the *P* value was 0.11 for the category 31 to 59 years and 0.32 for the category 60 years or older, showing that in both age categories there was no association between the pair of variables analyzed. Regarding chronic disease, the exposure group consisted of the participants without any comorbidity, so the *P* value of 0.07 for the category of those with at least one chronic disease showed no association between the two variables.

CONCLUSION

A temporal pattern of IgG response was not observed, but it is suggested that immunological memory is weak and there is no association between IgG production and age or chronic disease in mild COVID-19.

Key Words: Humoral response; Immunoglobulin G antibody; Immune memory; Mild cases COVID-19; SARS-CoV-2 infection; Serological test

Core Tip: This study suggests that no precise temporal pattern of humoral immunoglobulin G (IgG) response could be established. This fact suggests the absence of a robust immunological memory in mild cases of coronavirus 2019 disease, and furthermore, due to the lack of association between IgG response and age group, in mild cases of the disease the elderly do not appear to be a risk group for infection.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19), a disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China, in December 2019 and spread worldwidevery quickly, causing an unprecedented pandemic that impacted healthcare systems, the economy, politics, and social organization. Patients with COVID-19 may be asymptomatic or present with critical illness, and with symptoms including fever, cough, sore throat, malaise and myalgia. Some patients may experience gastrointestinal symptoms such as anorexia, nausea, and diarrhea[1].

Asymptomatic patients can be assumed to be uninfected, and thus they can be the focus of new outbreaks of the infection by transmitting the virus to healthcare workers or individuals with risk factors[1,2]. According to some studies, risk factors for complications of COVID-19 include advanced age, cardiovascular disease, chronic lung disease, diabetes, obesity and immunosuppression[3-7].

Therefore, the influence of comorbidities on the immune response profile and disease susceptibility has been widely discussed. The main comorbidities involved in this study are diseases of the immune system, such as asthma, rheumatoid arthritis (RA) and autoimmune gastritis (AIG). Although patients with severe asthma have been associated with a higher risk of COVID-19-related death[8], studies have indicated that the disease was not statistically associated with increased risk of infection or being hospitalized due to the disease[9,10].

Regarding the gastrointestinal system diseases, specifically AIG, the data in the literature indicate no relationship between autoimmunity and increased susceptibility to SARS-CoV-2[11]. On the other hand,

in relation to RA, a study of patients with rheumatic diseases (91.89% were autoimmune with 72.97% of RA, and 18.92% of systemic lupus erythematosus) showed that cardiovascular manifestations caused by COVID-19 appeared more frequently in patients with rheumatic diseases[12].

According to the Chinese data, 81% of individuals infected with SARS-CoV-2 developed mild or moderate COVID-19, 14% developed severe disease, and only 5% developed critical disease[13]. Therefore, it is critical to study asymptomatic cases and those with mild symptoms, as they represent a risk of COVID-19 spreading. One example is the infection of 71 individuals originated from an asymptomatic case in China[14]. Furthermore, it has been evidenced that mild cases of COVID-19 also cause an increase in the need for primary health care, potentially lasting up to 3 mo[15], which can further burden the health care system.

Another point that emphasizes the importance of studying mild cases is the sequelae left by the virus. SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) as a functional receptor to invade host cells. This enzyme's main function is the regulation of angiotensin 2 and is highly expressed in the lungs, intestine and kidneys[16]. This gives the pathogen a high capacity of dissemination[16,17]. Thus, although the virus mainly infects the respiratory system, its systemic dissemination can occur, affecting several organs[17].

As respiratory sequelae, besides lung damage, the main consequences observed were alterations in taste and smell, such as anosmia, hyposmia, ageusia or dysgeusia[17,18]. Such symptoms were mainly associated with the appearance of symptomatic manifestations of COVID-19, since the gateway of the virus into the body are both the oral and nasal cavities, the sites whose epithelial tissue presents receptors for these senses[17].

Furthermore, one study demonstrated the high presence of ACE2 in biopsies of the olfactory mucosa, and the enzyme is mainly present in Bowman's glands[16]. In addition, other reported sequelae involve the cardiovascular system, such as heart attack and pulmonary thromboembolism, as well as kidney disease, liver damage, and even neurological changes[17].

As for the differences in immune response between mild and severe cases of COVID-19, it was observed that patients with critical illnesses showed a higher and earlier immunoglobulin G (IgG) and immunoglobulin A (IgA) response against SARS-CoV-2, as well as high viral neutralization. On the other hand, 75% of patients with mild symptoms developed antibodies and these showed low or even no viral neutralization rate[19,20].

Accordingly, this study set out to evaluate the temporal pattern of IgG anti-SARS-CoV-2 response in mild cases of COVID-19. To this end, the study analyzed data from patients presenting with the disease between September 2020 and January 2021, prior to the introduction of vaccines.

MATERIALS AND METHODS

Population study and data collection

This study was approved by the Ethics Committee for research with humans of the Setor de Ciências da Saúde-Universidade Federal do Paraná (UFPR)/Brazil (Protocol no. 35872520.8.0000.0102) and all participants signed an informed consent form. Volunteers older than 18 years (n=393) were tested for COVID-19 by real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) and 191 were positive. Blood samples and clinicoepidemiological data from the volunteers were collected from November 2020 to February 2021. The clinicoepidemiological data were collected by means of a semi-structured questionnaire. Venous blood was collected from each volunteer in a tube without anticoagulant and the serum was separated by centrifugation at 2000 rpm for 2 min. At this time, COVID-19 vaccines were not yet being applied in Brazil. The 191 individuals diagnosed as being positive for COVID-19 by RT-qPCR were included in this study; all were residents of the municipality of Toledo/Paraná/Brazil. Serum samples were subjected to two different serological tests by enzyme-linked immunosorbent assay, a commercial test-Allserum EIA COVID19 IgG (MBiolog Diagnostics) and a test developed by UFPR/Setor Litoral[21]. Both tests detected anti-nucleocapsid IgG, the secondary antibody was bound to Horseradish peroxidase, chromogenic substrate was Tetramethylbenzidine and absorbance reading was performed at 450 nm. All assays were performed in duplicate and the manufacturers' recommendations were strictly followed; assays were analyzed by UV/Vis Multiskan Sky spectrometer (Thermo Fisher Scientific Inc.).

Statistical analysis

A database containing clinicoepidemiological characteristics and serological results was prepared. To perform multiple logistic regression, variables were selected by applying the $P < 0.05$ adjusted odds ratio criterion and using the Maximum likelihood estimation. The final model was obtained after testing for all possible multiple interactions with subsequent verification of model fit by the Hosmer & Lemeshow method. A receiver operating characteristic (ROC) curve was done to evaluate the ability of the model created to represent reality.

RESULTS

Serum samples from the volunteers spanned a time interval of less than 1 mo up to 7 mo between positive diagnosis for COVID-19/symptoms onset and serological testing for specific IgG. The majority of study participants (62.83%) had detectable IgG for SARS-CoV-2, of which 37.17% had no antibodies at the time of blood collection. Furthermore, as shown in Figure 1, regarding the residence time of IgG detectable by serological testing, only 1 volunteer (0.52%) was IgG positive for 7 mo. The time periods where the highest number of participants with detectable IgG was observed were 1, 2 and 3 mo, with 46 (24%), 25 (13.08%) and 19 (9.94%), respectively. Twelve participants (6.28%) had IgG antibodies at the end of 4 mo, 4 participants (2.09%) remained IgG positive at the end of 5 mo, 8 (4.19%) had IgG for 6 mo, and 5 (2.61%) were IgG positive for less than 1 mo.

Figure 1 also shows that 18 individuals (9.42%) no longer had detectable anti-SARS-CoV-2 IgG antibodies one month after being infected with SARS-CoV-2 and 3 (1.57%) were negative at less than 1 mo. Seventeen volunteers (8.90%) had no detectable anti-SARS-CoV-2 IgG after 2 mo; the same was observed with 16 volunteers (8.37%) at the end of 3 mo and 9 participants (4.71%) at the end of 4 mo. At 5 mo, 6 volunteers (3.14%) had negative anti-SARS-CoV-2 IgG, and at 6 or 7 mo 1 volunteer (0.52%) had no detectable IgG.

As shown in Table 1, regarding the period of time elapsed between diagnosis by RT-qPCR/symptoms onset and the date of blood collection for the study, no statistical significance was observed between the variables.

Regarding age, most participants (115 or 60.21%) were between 31 and 59 years old; 57 (29.84%) were between 18 and 30 years old, and 19 (9.95%) participants were 60 years old or older. Considering the age group between 31 and 59 years as the exposed group, the *P* value was 0.11 for the age group of 31 - 59 years and 0.32 for the group of 60 years or older, showing that there was no association between the pair of variables analyzed.

About the presence of chronic diseases, 135 (70.68%) participants had no comorbidity. On the other hand, 45 (23.56%) individuals had one chronic disease, while 10 (5.24%) had two and one (0.52%) participant had three. Regarding this variable, the exposure group considered was the participants without any comorbidity, so that the *P* value of 0.07 for those with at least one chronic disease showed no association between the two variables studied.

The statistical model showed adequate fit evaluated by the Hosmer & Lemeshow method ($\chi^2 = 3.656$; GL = 8; $P = 0.887$). The area under the ROC curve showed that the estimated probability model could predict approximately 67.59% of the factors associated with the outcome.

DISCUSSION

The viral proteins play a striking role in diagnosing COVID-19 and monitoring the production of antibodies against the coronavirus by serological tests. In this regard, tests for COVID-19 can be either nucleic acid amplification tests (RT-qPCR) or serological tests. While RT-qPCR is recommended for active coronavirus infection, serological tests are recommended for antibody response [immunoglobulin M (IgM) and IgG][22]. Thus, serologic testing and antibody analysis are useful to verify whether there has been previous exposure to the virus and to quatify the patient's humoral immunity levels and types of antibodies produced[23].

Therefore, the production of specific antibodies is essential, since they are responsible for effective protection against the severe forms of the disease, even though there are other cells, such as TCD^{4+} and TCD^{8+}, that act in the immunity process[24]. In addition, IgM antibodies provide the first line of defense during infections, while IgG production provides immunity and long-term memory[25].

The level of antibody production depends on the elapsed time of infection, the severity of the disease, the viral load to which the patient has been exposed, and individual patient characteristics such as age, sex, and pathogen elimination[26]. There may also be variations in the detection of antibody levels according to the sensitivity of the serological test used.

Regarding the elapsed time of infection, while IgM antibodies can be detected at about five days of infection and reach higher rates between two and three weeks of illness, IgG antibodies begin to be produced about 14 d after the symptoms onset and individuals with more severe disease have higher antibody levels[27,28]. Regarding COVID-19, the observation period for most studies on the production of specific anti-SARS-CoV-2 antibodies is 12 wk, and it is still unclear how antibody titers may change in subsequent periods[29].

In a study conducted in Wuhan, China, after confirmation of coronavirus infection by RT-qPCR, asymptomatic individuals were recruited to detect levels of anti-SARS-CoV-2 antibodies. Of a total of 63 individuals with asymptomatic infections, 38.1% (24 patients) produced no antibodies and 61.9% (39 patients) produced only small titers. Six (11.8%) out of 51 patients with mild symptoms, produced no antibodies and 88.2% (45 patients) produced higher levels of antibodies when compared to asymptomatic patients.

Table 1 Temporal pattern of immunoglobulin G and baseline characteristics of real-time reverse transcriptase-polymerase chain reaction positive individuals for coronavirus disease 2019

Variable		*n*	%	*P* value	OR adjusted	95%CI
IgG						
	Negative	71	37.17			
	Positive	120	62.83			
Age						
	18 to 30 yr	57	29.84			
	31 to 59 yr	115	60.21	0.11	1.26	(0.89-3.48)
	60 yr or +	19	9.95	0.32	1.92	(0.60-6.60)
Time						
	< 1 mo	8	4.19	0.09	0.79	(0.16-3.83)
	1 mo	64	33.51			
	2 mo	42	21.99	0.18	0.56	(0.24-1.30)
	3 mo	35	18.32	0.13	0.50	(0.20-1.22)
	4 mo	21	10.99	0.36	0.61	(0.21-1.75)
	5 mo	10	5.24	0.09	0.29	(0.07-1.21)
	6 mo	9	4.71	0.25	3.55	(0.41-30.69)
	7 mo	2	1.05	0.39	0.27	(0.01-5.14)
Chronic disease						
	Not	135	70.68			
	Yes	56	29.32	0.07	2.03	(0.94-4.40)
Number of chronic diseases						
	0	135	70.68			
	1	45	23.56			
	2	10	5.24			
	3	1	0.52			

IgG: Immunoglobulin G; CI: Confidence interval; OR: Odds ratio.

In the same study, in asymptomatic individuals, antibody production started seven days after exposure, peaked between 10 and 25 d, and decreased rapidly thereafter. On the other hand, in individuals with mild symptoms, one day after the onset of symptoms, antibodies were already produced, even at a low level, and titration increased persistently up to 22 d, maintaining high levels for at least 65 d[30].

In a study of 164 participants in Singapore, 19 patients (12%) did not develop neutralizing antibodies against SARS-CoV-2; 44 patients (27%) produced antibodies early (approximately 20 d after symptoms onset), but disappeared in less than 180 d; 46 patients (28%) had neutralizing antibodies for more than 180 d after symptoms onset; 52 patients (32%) had minimal decay of neutralizing antibodies; and three patients (2%) who had increased neutralizing antibodies 90 d after symptoms onset[31].

Similarly, 140 patients with COVID-19 positive for RT-qPCR were recruited for a study in France, of whom 44 were admitted to the intensive care unit (ICU), 42 were hospitalized without the need for ICU, and 54 received outpatient treatment only (including eight asymptomatic cases). It was observed that most patients in the different groups produced neutralizing antibodies, but the neutralizing activity was variable, *i.e.* higher in the group of patients admitted to the ICU, so that only one patient in this group did not develop a neutralizing antibody response at the time of collection. In contrast, 21.9% of hospitalized patients and 25% of outpatients treated did not develop neutralizing antibodies at the time of the study[32].

This study supports the hypothesis that seroconversion is observed more frequently in individuals with severe symptoms and that they have higher antibody titers than mild and asymptomatic cases. This means that plasma titers are approximately eight times higher for severe cases[33].

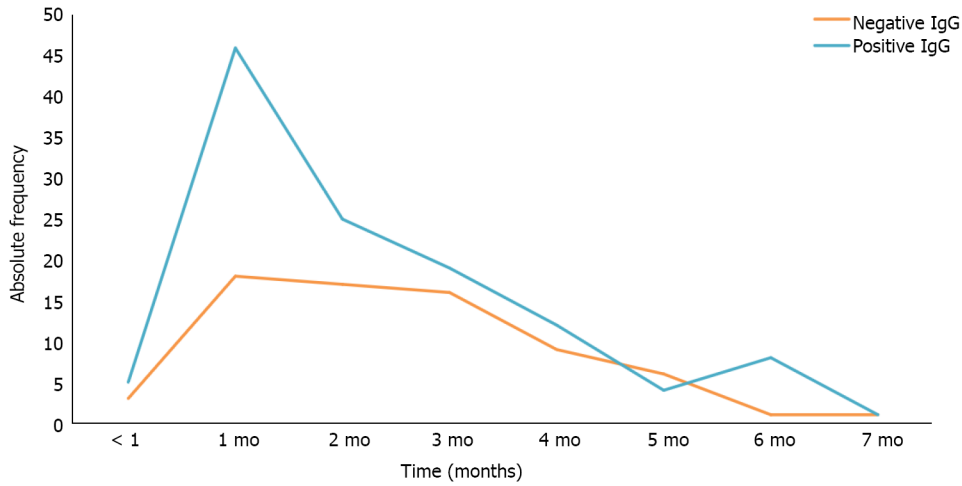

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Figure 1 Correlation between positive or negative immunoglobulin G in volunteers and the time after diagnosis of coronavirus disease 2019/symptoms onset. IgG: Immunoglobulin G.

Therefore, this study focused on understanding humoral immunity in mild cases of COVID-19 and regarding the time period of IgG detection in the serum of patients, similar results were obtained to previous studies. This finding contributes and adds data to the current knowledge about the humoral response against mild COVID-19 as it is a study conducted in a Western country with a larger sample size.

However, the temporal profile of the antibody response raised a concern by other authors[34-36]. Humoral immunity against SARS-CoV-2 does not appear to be durable, especially in individuals with mild symptoms or those who are asymptomatic, which make up the majority of COVID-19 cases. This fact is corroborated by the several doses of the SARS-CoV-2 vaccine, which is currently in its fourth dose in Brazil. On the other hand, it was also found that individuals with low titers or even undetectable levels of neutralizing antibodies can still be protected from subsequent infections, considering that memory B cells are still present in recovered patients[37,38].

The effect of age on immunity against SARS-CoV-2 has been widely discussed. A cohort study developed by the University of Virginia analyzed the antibody responses in individuals who received two doses of the vaccine BNT162b2 (Pfizer®) or mRNA-1273 (Moderna®) and had a blood sample collected seven to 31 d after the second dose. The results showed that participants aged 50 years and older who received BNT162b2 had lower pre-boost IgG levels than participants younger than 50 years who received the same vaccine. Individuals aged 50 years and older who received BNT162b2 had post-boost IgG levels that were also lower than levels found in younger participants[39].

Another study examined the immune response in elderly participants and younger healthcare professionals following immunization with the BNT162b. The results showed that after the first dose of the vaccine, IgG or IgA levels were lower in older individuals. In addition, elderly participants showed lower interferon-γ and interleukin (IL)-2 production by T cells specific against SARS-CoV-2 when compared to younger individuals[40].

Furthermore, a cohort study conducted in Greece analyzed the IgG response against the protein S of SARS-CoV-2 in a group of individuals after immunization with two doses of the BNT162b2 vaccine. The results revealed that younger patients (21-30 years old) had the highest antibody levels in both periods [41].

On the other hand, some studies have observed that older patients have been related to higher levels of antibodies against SARS-CoV-2. A study from Union Hospital (Huazhong University of Science and Technology, Wuhan, China) conducted with convalescent patients identified the presence of anti-SARS-CoV-2 antibodies one year after infection, in addition to a difference in IgG response according to the age of the patients. It was observed that the mean IgG antibody level was relatively low in younger convalescent patients (aged 21 - 35 years), and gradually increased to about 60% in older patients. The mean anti-SARS-CoV-2 IgG level was significantly higher in patients older than 35 years when compared to those younger than or equal to 35 years[42]. In our study, however, we found no statistical significance in the association between age and anti-SARS-CoV-2 IgG antibody levels. Seroconversion in the group of older individuals was not lower when compared to younger participants, demonstrating that age is possibly not a risk factor when analyzing mild cases of COVID-19.

Regarding the association between COVID-19 and pre-existing comorbidities, those with the highest association are hypertension and obesity, followed by metabolic disease, cardiovascular disease, neurological disease, chronic lung disease, kidney disease, asthma, immunosuppression, gastrointestinal or liver disease, and finally autoimmune disease[43]. According to some authors, COVID-19

has also been associated with type 2 diabetes mellitus (DM2), cancer, and chronic kidney disease[44].

It has been found in numerous studies that individuals with DM2 may have higher severity and mortality from COVID-19. This fact is due to the existing inflammatory condition in these patients, with higher levels of pro-inflammatory molecules, such as cytokines, especially IL-6. In addition, the presence of DM2 causes the response to SARS-CoV-2 to show a large amount of interferon and a delayed Th1 and Th17 response, which contributes to a more intense inflammatory response[45]. An example of this is a study showing that increased viral replication and production of pro-inflammatory cytokines may be related to high glucose concentration[46].

Coronaviruses bind to ACE2, reducing the activity of this receptor and increasing vascular permeability[47]. However, in individuals with systemic arterial hypertension (SAH) and DM, there is a higher number of these receptors when compared to the general population, which may explain the more severe cases of COVID-19 in these patients. Furthermore, SARS-CoV-2 produces endothelial injury, causing an inflammatory vascular state, pro-coagulant state, and a cellular infiltrate, also clarifying the more severe symptoms in individuals with these chronic diseases[48,49].

In addition, the presence of SAH also determines a pro-inflammatory state, arising from the endothelial dysfunction caused by this disease, leading to excessive activation of coagulation and platelets, in addition to the production of cytokines, antimicrobial peptides, and reactive oxygen species. This excessive activation may not only cause damage to the respiratory epithelium, but also reduce lung function and increase the local inflammatory response, contributing to further occurrence of complications from COVID-19[50,51].

An important question of the study is how COVID-19 affects patients with autoimmune diseases, such as RA. In a study of 11 122 individuals with COVID-19, patients with RA were found to have a higher chance of hospitalization or death than healthy individuals, and the study used an unadjusted model. However, when adjusting for age, sex, and comorbidities, no greater chance of unfavorable outcomes was observed[51]. Thus, AR is associated with a higher risk of infection and death in patients with COVID-19 when taking into account active AR, the presence of other diseases and the use of medications such as Rituximab, sulfasalazine or other immunosuppressive drugs[52].

Regarding the association between AIG and COVID-19, a study conducted at the Foundation of San Matteo Hospital, Italy, analyzed the susceptibility to COVID-19 in 400 drug-free immunosuppressive patients with autoimmune diseases, 100 of whom had AIG. The findings showed that among the individuals with AIG, seven (7%) had already tested positive for COVID-19, one (1%) required hospitalization for COVID-19, and 43 (43%) were vaccinated for SARS-CoV-2.

Furthermore, considering all investigated autoimmune diseases, molecular nasopharyngeal swabs and/or serology for SARS-CoV-2 testing showed that 33 (8.2%) tested positive[53]. These data are similar to those reported in the general population in the same geographical area in Italy[53], suggesting that the risk of COVID-19 in individuals with autoimmune diseases appears to be the same as in the general population.

Asthma is still being studied as a risk factor for COVID-19. The proposed hypothesis that the occurrence of more severe complications caused by COVID-19 in patients with asthma is due to a possible interaction between the pathobiology of SARS-CoV-2 and asthma. Thus, since the virus causes an intense inflammatory response and asthmatic individuals already have narrowed airways with high mucus production, pneumonia caused by the virus can lead to severe complications[54]. However, asthma can also lead to favorable outcomes, since it induces a negative regulation of ACE2, an enzyme that assists in the process of viral entry of SARS-CoV-2 into lung tissue[55]. On the other hand, a meta-analysis study did not identify a statistically significant increase in mortality and a worse prognosis for COVI-19 in asthmatic individuals[9].

Thus, although there are studies indicating the need for more intensive treatment in individuals with DM2, SAH, and other comorbidities[56], in patients with mild symptoms, this association does not seem to materialize, as the presence of previous diseases was not statistically significant for seroconversion in our study participants.

CONCLUSION

This study suggests that in mild cases of COVID-19, it is not possible to establish a temporal relationship of specific IgG production, raising the hypothesis that such a relationship may, in fact, not exist or that perhaps there is more than one type of relationship since there is interference from several factors, such as age, sex, presence of comorbidities, viral elimination, viral load, among others. It is also suggested that the virus generates a weak and non-lasting immune response in mild cases. Furthermore, a lower production of IgG antibodies was not observed in the elderly and in individuals with previous chronic diseases, leading to the conclusion that in mild cases of COVID-19, these patients may not be a risk group for unfavorable outcomes when analyzing the humoral response.

ARTICLE HIGHLIGHTS

Research background

The molecular test used in the diagnosis of coronavirus disease 2019 is very specific and sensitive, however, it is not able to detect previous exposure to the virus nor to assess immunological memory. Therefore, serological tests that have this capability are used as tools for understanding the course of the humoral immune response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Research motivation

The motivation for this study arose from the serological test developed at the Federal University of Paraná, which in the validation process showed better sensitivity than commercial tests. A more sensitive test allows specific antibodies to be detected even at low titers, and thus to effectively assess whether there is still a protective antibody response in individuals who have been infected by the virus.

Research objectives

The aim of this study was to identify if a pattern of SARS-CoV-2 specific immunoglobulin G (IgG) production can be determined according to the time elapsed since diagnosis of the disease/onset of symptoms. The data could indicate, for example, the interval between vaccination doses.

Research methods

This study was initiated after approval by the ethics committee. The participants were tested by real-time reverse transcriptase-polymerase chain reaction, the municipal government provided us with the data. Only positive cases were included in the study. Blood collection was performed by our research team and the method used for specific IgG antibodies was the indirect enzyme-linked immunosorbent assay. Statistical analyses were performed by the statistician of the research group, one of the authors of the manuscript.

Research results

The results of the study showed that there is no time pattern for the production of specific IgG. Less than one month after infection, some participants no longer have detectable IgG in the serum, while others have the antibodies seven months after infection.

Research conclusions

In addition to the impossibility of establishing a temporal pattern of IgG response, the data indicate that SARS-CoV-2 does not appear to induce a long-lasting humoral response.

Research perspectives

The study perspective is to analyze the immunoglobulin M (IgM) response of the same volunteers and determine the titers of both IgG and IgM to better understand seroconversion and the robustness of the anti-SARS-CoV-2 antibody response.

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FOOTNOTES

Author contributions: Teixeira KN and Brandalize APC designed, coordinated the study and interpreted the data; Pilati Campos IM and Peiter GC carried out the experiments, acquired and analyzed data; dos Santo MB carried out the statistical analyses; Pilati Campos IM and Marques M reviewed the literature and wrote the manuscript; Teixeira KN and de Melo FF reviewed the manuscript.

Institutional review board statement: The study was approved by the Ethics Committee for research with humans of the Setor de Ciências da Saúde-Universidade Federal do Paraná (UFPR)/Brazil (Protocol no. 35872520.8.0000.0102).

Informed consent statement: All study participants were over 18 years old, and they read and signed the informed consent statement.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The study participants gave informed consent for data disclosure in an anonymous way,

without exposing their identity.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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ORIGINAL ARTICLE

Observational Study

Correlation of serum SARS-CoV-2 IgM and IgG serology and clinical outcomes in COVID-19 patients: Experience from a tertiary care centre

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Abstract

BACKGROUND

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus has become a pandemic for the last 2 years. Inflammatory response to the virus leads to organ dysfunction and death. Predicting the severity of inflammatory response helps in managing critical patients using serology tests IgG and IgM.

AIM

To investigate the correlation of the serology (IgM and IgG) with reverse transcriptase polymerase chain reaction (RT-PCR) status, disease severity [mild to critical], intensive care unit (ICU) admission, septic shock, acute kidney injury, and in-hospital mortality.

METHODS

We conducted a longitudinal study to correlate serum SARS-CoV-2 immunoglobulin M (IgM) and immunoglobulin G (IgG) serology with clinical outcomes in coronavirus disease 2019 (COVID-19) patients. We analyzed patient data from March to December 2020 for those who were admitted at All India Institute of Medical Sciences Rishikesh. Clinical and laboratory data of these patients were collected from the e-hospital portal and analyzed. A correlation was seen with clinical outcomes and was assessed using MS Excel 2010 and SPSS software.

RESULTS

Out of 494 patients, the mean age of patients was 48.95 ± 16.40 years and there were more male patients in the study (66.0%). The patients were classified as mild-moderate 328 (67.1%), severe 131 (26.8%), and critical 30 (6.1%). The mean duration from symptom onset to serology testing was 19.87 ± 30.53 d. In-hospital mortality was observed in 25.1% of patients. The seropositivity rate (*i.e.*, either IgG or IgM > 10 AU) was 50%. IgM levels (AU/mL) (W = 33428.000, $P \leq 0.001$) and IgG levels (AU/mL) (W = 39256.500, $P \leq 0.001$), with the median IgM/ IgG levels (AU/mL), were highest in the RT-PCR-Positive group compared to RT-PCR-Negative clinical COVID-19. There was no significant difference between the two groups in terms of all other clinical outcomes (disease severity, septic shock, ICU admission, mechanical ventilation, and mortality).

CONCLUSION

The study showed that serology levels are high in RT-PCR positive group compared to clinical COVID-19. However, serology cannot be useful for the prediction of disease outcomes. The study also highlights the importance of doing serology at a particular time as antibody titers vary with the duration of the disease. In week intervals there was a significant correlation between clinical outcomes and serology on week 3.

Key Words: Inflammatory response; Reverse transcription polymerase chain reaction; SARS-CoV-2; Serology IgM and IgG

Core Tip: Coronavirus disease 2019 (COVID-19) serology levels are high in reverse transcriptase polymerase chain reaction positive group compared to clinical COVID-19. However, serology cannot be useful for the prediction of disease outcomes. The study also highlights the importance of doing serology at a particular time as antibody titres vary with the duration of the disease. In week interval there were significant correlation with clinical outcomes and serology on week 3.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) has affected almost 581 million people with around 6.4 million deaths as of July 2022 [World Health Organization (WHO)][1]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can infect individuals from different age groups and causes a wide spectrum of disease manifestations ranging from asymptomatic, to mild, moderate to severe symptoms with possible fatal outcomes[2]. Age, sex, pre-existing comorbidities, host genetics as well as host immune response are the key factors determining the outcomes[3]. The reverse transcriptase polymerase chain reaction (RT-PCR) assay is the right method to diagnose SARS-CoV-2. Unfortunately, the sensitivity of the RNA test in the real world is not satisfactory and, false-negative and false-positive cases have also been reported owing to several factors[4]. According to recent WHO case definitions, the RT-PCR negative patients who meet clinical and epidemiological criteria or patients with severe acute respiratory illness who have typical chest imaging features or unexplained anosmia or ageusia are termed as probable COVID-19 patients, better term would be RT-PCR-negative clinical COVID-19[5,6].

Serological tests are increasingly applied for the diagnosis of SARS-CoV-2 infection, though not evidenced by various guidelines. Blood levels of immunoglobulin SARS-CoV-2 immunoglobulin G (IgG) & immunoglobulin M (IgM) are also deployed for evaluating immune responses and confirming the diagnosis in symptomatic patients presenting outside the window of positivity for RT-PCR-based SARS-CoV-2 testing[7]. Few studies have assessed the utility of seroconversion profiles to predict infection severity or outcomes following SARS-CoV-2 infection. A strong association was observed between the magnitude of antibody response and patient survival, disease severity, and fatal outcomes [8]. Furthermore, several studies have documented discrepancies in findings related to the timing of SARS-CoV-2 antibody seroconversion and the onset of symptoms[9-11]. More information about the dynamics of the early humoral immune response is needed to realize the full potential of serological

testing for SARS-CoV-2. The dynamics of antibody responses, in COVID-19 patients with different clinical presentations, are still not well-characterized. Such information can help our understanding of the nature of COVID-19 infection and guide patient management.

Here, we studied the seropositivity and kinetics of SARS-CoV-2 IgM and IgG antibodies in blood samples collected between 2 to 85 d post-symptoms onset from a cohort of 493 COVID-19 patients. The objectivity was the correlation of the serology (IgM and IgG) with RT-PCR status, disease severity (mild to critical), intensive care unit (ICU) admission, septic shock, acute kidney injury (AKI), and in-hospital mortality.

MATERIALS AND METHODS

Study design and setting

The study was an observational longitudinal study conducted on COVID-19 patients admitted to a tertiary care hospital, All India Institute of Medical Sciences (AIIMS), Rishikesh, India from August 2020 to November 2020. The study was designed according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.

Inclusion criteria

COVID-19 patients with detectable SARS-CoV-2 RNA in respiratory samples since disease onset. Clinical COVID-19 patients i.e. cases with clinical manifestations characteristic of COVID-19 but with negative SARS-CoV-2 RT-PCR test from admission until discharge[1,2]. Patients of both genders with age ≥ 15 years. Patients with complete data on serological results available in files.

Exclusion criteria

Patients not fulfilling COVID-19 diagnostic criteria as per institutional protocol. Asymptomatic patients, pregnant women, and patients having incomplete data.

Case definitions

COVID-19 Severity classification: Patients were classified as mild, moderate, severe, and critical according to the WHO guidelines[1].

Serological tests

iFlash-SARS-CoV-2 (Shenzhen Yhlo Biotech Co. Ltd.), a paramagnetic particle-based chemiluminescent immunoassay (CLIA) was used for the determination of IgM and IgG antibodies against SARS-CoV-2 nucleocapsid protein and spike protein. According to the manufacturer's inserts [V1.0 English Fd. 2020–02-20], the IgM and IgG cut-off is 10 AU/mL, *i.e.*, an antibody titer above titer over 10 AU/mL was regarded as positive.

Treatment of patients

Patients were treated uniformly as per institutional guidelines.

Participants' enrolment

All COVID-19 admitted patients at All India Institute of Medical Sciences, Rishikesh during the above period.

Variables and outcome and data collection

Full information regarding demographic characteristics, the time course of symptoms, time of presentation and testing, presenting symptoms, final diagnosis, treatments received [*i.e.* oxygen therapy, corticosteroids, ICU admission, invasive ventilation requirement, and dialysis] were collected in master excel. The medical records were further critically reviewed for important missed data.

Study size

All consecutive patients during the above period.

Ethics

The Approval for this study was obtained from the institute ethics committee of AIIMS Rishikesh with approval no CTRI/2020/08/027169.

Statistical methods

All the statistical analyses were performed using the statistical package for social sciences (SPSS), Windows version 23 software package (SPSS, CHICAGO, IL, United States). Non-normally distributed continuous variables were presented as medians [interquartile ranges (IQR)]. Differences between non-

normally distributed continuous variables were assessed using the Mann–Whitney U test. Categorical variables were presented as counts (%). Differences between categorical variables were assessed using the χ^2 or Fisher's exact tests. A two-sided value of $P < 0.05$ was considered statistically significant.

Bias

As all patients sampling for IgG and IgM was conducted only once, and time to sampling may be an important variable that can confound the study results, we analyzed the association between different clinical outcomes and its association with IgG and IgM levels in a time-dependent manner based on the time interval between symptom onset and IgM and IgG testing. We used Bayesian latent class modeling for the evaluation of the diagnostic performance of RT-PCR, IgM, and IgG tests in COVID-19.

RESULTS

Demographic characteristics

A total of 494 hospitalized patients were enrolled in the study, among them 199 were RT-PCR positive and 294 were clinically diagnosed COVID-19 patients (Table 1, Figures 1 and 2).

Seropositivity status among COVID-19 patients

In this cohort of 494 data on seropositivity was available for 455 patients, and the seropositivity rate (*i.e.* either IgM or IgG > 10 AU) was 247 (54%). Out of these IgM seropositivity was observed in 103/455 (22.63%) and for IgG 224/455 (49.01%). IgM or IgG seropositivity increased to a peak at week 4 and then decreases after 4 wk (> 28 d, Figure 3).

Association between COVID-19 serology and RT-PCR status

There was a significant difference between the 2 groups in terms of IgM levels (AU/mL) (W = 33428.000, $P \leq 0.001$) and IgG levels (AU/mL) (W = 39256.500, $P \leq 0.001$), with the median IgM/ IgG levels (AU/mL) being highest in the RT-PCR-Positive group. In all weeks, there was a significant difference between the 2 groups except for week 4 (22-28 Days) there was no significant difference in terms of IgM and IgG levels (AU/mL) (Figure 4).

Association between COVID-19 serology and disease severity

There was no significant difference between the groups in terms of IgM levels (AU/mL) ($\chi^2 = 2.975$, $P = 0.395$) and IgG levels ($\chi^2 = 2.463$, $P = 0.482$). In week 3, there was a significant difference between the groups in terms of IgM Levels (AU/mL) ($\chi^2 = 7.732$, $P = 0.021$) and IgG levels (AU/mL) ($\chi^2 = 7.707$, $P = 0.021$), with the median IgM and IgG levels (AU/mL) being highest in the critical group. In all the other weeks, there was a significant difference between the 2 groups in terms of IgM and IgG levels (AU/mL) (Supplementary Figure 1).

Association of COVID-19 serology with acute respiratory distress syndrome types and Oxygen requirement

There was a significant difference between the 4 groups in terms of IgM Levels (AU/mL) ($\chi^2 = 7.985$, $P = 0.046$) and IgG levels (AU/mL) ($\chi^2 = 8.501$, $P = 0.037$). The median IgM levels (AU/mL) were highest in the mild acute respiratory distress syndrome (ARDS) group and median IgG levels (AU/mL) were highest in the Moderate ARDS group.

In all weeks no significant difference between the groups in terms of IgM levels and IgG levels. However, in week 3 there was a significant difference between the 4 groups in terms of IgM levels (AU/mL) ($\chi^2 = 10.837$, $P = 0.013$) and IgG of IgG levels (AU/mL) ($\chi^2 = 9.682$, $P = 0.021$). The median IgM levels (AU/mL) were highest in the Mild ARDS group and the median IgG levels (AU/mL) were highest in the severe ARDS group.

There was a significant difference between the 3 groups in terms of IgM levels (AU/mL) ($\chi^2 = 6.795$, $P = 0.033$), with the median IgM levels (AU/mL) being highest in the Oxygen Therapy: < 6 L/min group. There was no significant difference between the groups in terms of IgG Levels (AU/mL) ($\chi^2 = 4.532$, $P = 0.104$).

There was a significant difference between the 3 groups in terms of IgM levels (AU/mL) in week 1 ($\chi^2 = 6.053$, $P = 0.048$), with the median IgM levels (AU/mL) being highest in the Oxygen Therapy: < 6 L/min group, week 2 ($\chi^2 = 6.392$, $P = 0.041$), with the median IgM levels (AU/mL) being highest in the Oxygen Therapy: > 6 L/min group and Week 3 ($\chi^2 = 6.283$, $P = 0.043$), with the median IgM levels (AU/mL) being highest in the Oxygen Therapy: < 6 L/min group. There was a significant difference between the 3 groups in terms of IgG levels (AU/mL) ($\chi^2 = 8.629$, $P = 0.013$), with the median IgG levels (AU/mL) being highest in the Oxygen Therapy: > 6 L/min group. In all other weeks no significant difference between the groups in terms of IgM levels and IgG levels (Supplementary Figure 2).

Table 1 Demographic table

Features	Median [Q1-Q3] or frequency [%]
Age (yr)	50.00 [36.00-61.00]
Gender	
Male	326 [66.0]
Female	168 [34.0]
IgG (AU/mL)	7.82 [0.63-57.07]
IgG	
< 10 AU/mL	231[50.99]
> 10 AU/mL	224 [49.01]
IgM (AU/mL)	0.96 [0.48-7.68]
IgM	
< 10 AU/mL	352 [77.37]
> 10 AU/mL	103 [22.8]
RT-PCR	
Positive	199 [40.4]
Negative	294 [59.6]
Onset-Testing Interval (d)	12.00 [7.00-21.00]

RT-PCR: Reverse transcriptase polymerase chain reaction.

Association of COVID-19 serology with Septic shock

There was no significant difference between the groups in terms of IgM Levels (AU/mL) (W = 1191.500, *P* = 0.168) and IgG levels (AU/mL) (W = 19537.500, *P* = 0.261).

In all weeks no significant difference between the groups in terms of IgM levels and IgG levels. However, there was a significant difference between the 2 groups in terms of IgM levels AU/mL (W = 1827.000, *P* = 0.035), with the median IgM levels (AU/mL) being highest in the no Septic Shock group. In week 3 IgG levels (AU/mL) (W = 317.000, *P* = 0.022), with the median IgG levels (AU/mL) being highest in the Septic Shock group and in > 4 wk (W = 366.000, *P* = 0.042), with the median IgG levels (AU/mL) being highest in the no Septic Shock group (Supplementary Figure 3).

Association of COVID-19 serology with the requirement of ICU admission

There was no significant difference between the groups in terms of IgM levels (AU/mL) (W = 23685.000, *P* = 0.668) and IgG (W = 25763.500, *P* = 0.157).

In all weeks no significant difference between the groups in terms of IgM levels and IgG levels. However, there was a significant difference between the 2 groups in terms of IgM levels (AU/mL) on week 3 (W = 403.500, *P* = 0.031) and IgG (W = 460.000, *P* = 0.038) with the median IgM levels (AU/mL) being highest in the group requiring ICU admission (Supplementary Figure 4).

Association of COVID-19 serology with the requirement of mechanical ventilation

There was no significant difference between the groups in terms of IgM levels (AU/mL) (W = 20744.500, *P* = 0.099) and IgG levels (AU/mL) (W = 23067.000, *P* = 0.460).

In all weeks no significant difference between the groups in terms of IgM levels and IgG levels. However, there was a significant difference between the 2 groups in terms of IgM levels (AU/mL) on week 2 (W = 2070.000, *P* = 0.035) and > 4 wk (> 28 d) (W = 358.500, *P* = 0.033), with the median IgM levels (AU/mL) being highest in the no Invasive Ventilation group (Supplementary Figure 5).

Association of COVID-19 serology with AKI and requirement of dialysis

There was no significant difference between the groups in terms of IgM Levels (AU/mL) (W = 23261.500, *P* = 0.425) and IgG levels (AU/mL) (W = 26023.500, *P* = 0.767).

In all weeks no significant difference between the groups in terms of IgM levels and IgG levels. However, there was a significant difference between the 2 groups in terms of IgM levels (AU/mL) on week 2 (W = 2473.000, *P* = 0.008), and IgG levels (AU/mL) (W = 2755.500, *P* = 0.043) with the median IgM/ IgG levels (AU/mL) being highest in the no Acute Kidney Injury group.

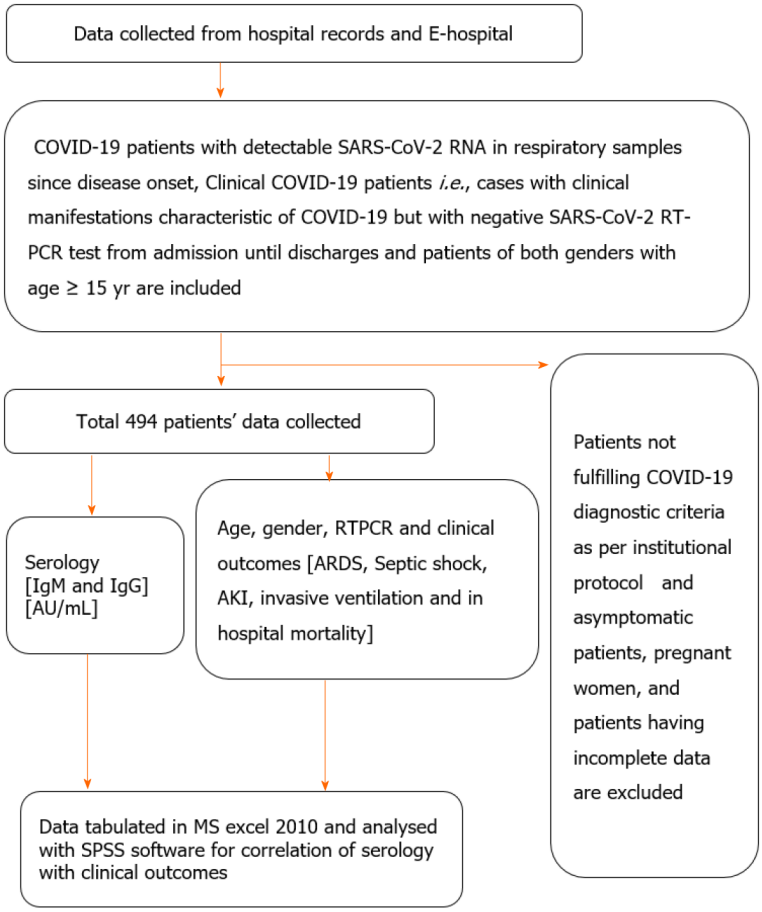

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Figure 1 Study flow chart. AKI: Acute kidney injury; ARDS: Acute respiratory distress syndrome; COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; IgG: Immunoglobulin G; IgM: Immunoglobulin M; RT-PCR: Reverse transcriptase polymerase chain reaction.

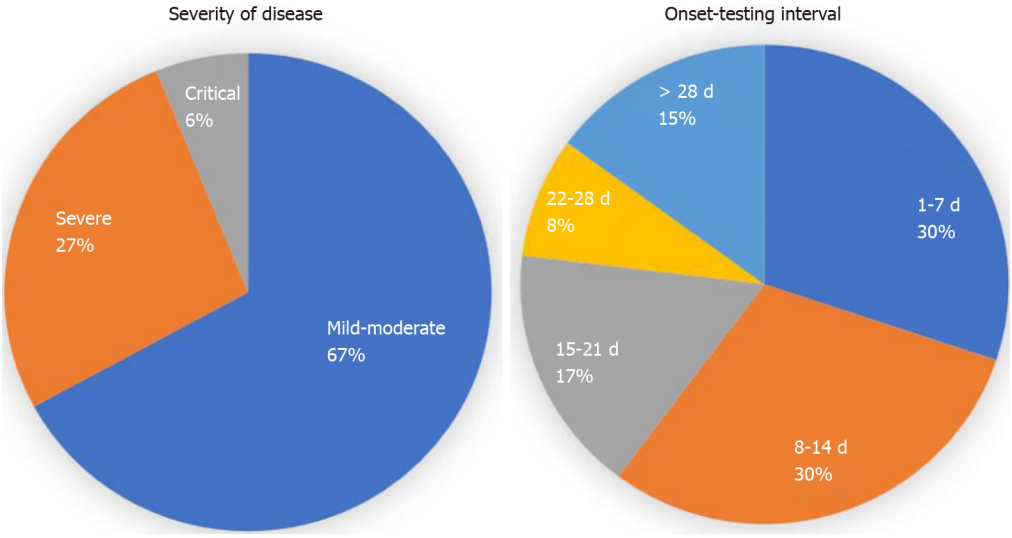

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Figure 2 Baseline demographic characteristic of patients.

There was a significant difference between the 2 groups in terms of IgM levels (AU/mL) (W = 14962.000, $P \leq 0.001$), with the median IgM levels (AU/mL) being highest in the no Dialysis group. However, there was no significant difference between the groups in terms of IgG levels (AU/mL) (W = 14553.000, $P = 0.206$). In all weeks no significant difference between the groups in terms of IgM levels and IgG levels (Supplementary Figure 6).

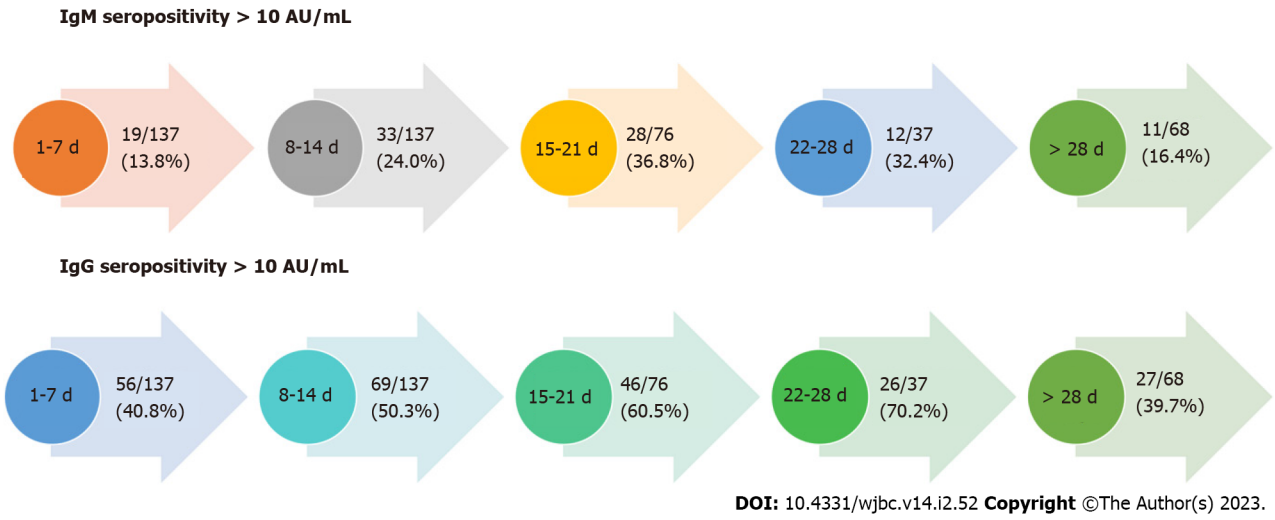

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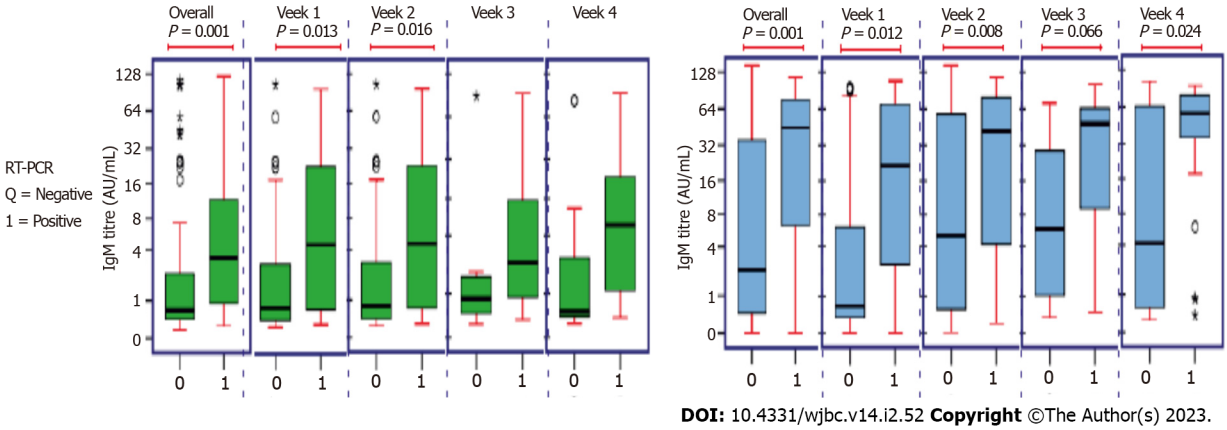
Figure 3 Seropositivity status with the duration of illness.



DOI: 10.4331/wjbc.v14.i2.52

Figure 4 Association of coronavirus disease 2019 serology and reverse transcriptase polymerase chain reaction status.

Association between COVID-19 serology and outcome: Survivor vs non-survivor

There was no significant difference between the groups in terms of IgM levels (AU/mL) (W = 21870.000, P = 0.058) and IgG levels (AU/mL) (W = 23088.500, P = 0.738).

In all the weeks there was no significant difference between the groups in terms of IgM levels and IgG levels. However, there was a significant difference between the 2 groups in terms of IgM levels (AU/mL) on week 4 (W = 136.500, P = 0.032) and > 4 wk (> 28 d) (W = 575.500, P = 0.003) with the median IgM levels (AU/mL) being highest in the survival group (Supplementary Figure 7).

DISCUSSION

The COVID-19 RT-PCR test is the most commonly used molecular test for the diagnosis of COVID-19 infection and is considered the gold standard test[12]. COVID-19 serology has emerged as one of the alternatives for diagnosing the COVID-19 disease. One of the meta-analyses by Chen *et al*[13] showed that the panel of IgG+ or IgM+ had a sensitivity of almost 79%, followed by IgG+ IgM+/- (73%), IgG+/- IgM+ (68%). Pooled specificities of these tests ranged from 98% to 100%. In our study also, in patients who had clinical COVID-19, almost 50% of patients were seropositive (IgM+ or IgG+).

Various studies have revealed that certain biochemical markers like IL-6 can be used as a prognostic marker for COVID-19[14]. The role of COVID serology in this aspect is less investigated upon. One of the retrospective studies done by Yan *et al*[15] showed that patients who had severe COVID-19 disease had higher COVID-19 IgG antibodies after 1 year. In this study also patients who were RT-PCR positive had statistically significant COVID-19 antibody serology. Also, Seropositivity for IgG increases as disease severity increases as shown in this study.

In one of the cross-sectional studies done in Iran, the study suggested that the patients who were IgG and IgM-positive had more severe symptoms compared to patients who had negative serology[16]. If we see the relationship between COVID-19 serology and complications, not many studies had been done in the past. This study had shown that patients who had higher COVID-19 IgG levels at three weeks had more severe ARDS and oxygen requirements compared to other patients. We also observed that there was a statistically significant difference in IgG antibody titers between the presence or absence of septic shock at three weeks. A similar trend was seen for ICU admissions and the need for mechanical ventilation. Also, in patients, who developed AKI there was more IgG seropositivity than IgM.

Previous studies by Liu *et al*[18], 2020, Zhang *et al*[19] showed that higher antibody (IgM and IgG) levels are seen in patients with severe and critical patients compared to mild-moderate patients[17-19]. Chen *et al*[20], 2021 study shows similar results as the above studies. However, the study showed antibody titer levels may vary and higher antibody titers were present in some mild-moderate category patients than in severe and critical patients. These findings are due to variations in serology to symptom onset interval[11,20,21]. The study also did not find a statistically significant correlation between antibody tires with AKI, mechanical ventilation, ICU requirement, septic shock, and mortality.

This study shows that higher body titers are associated with poor outcomes at a particular time serology to symptom onset interval. There are some limitations in this study first, it is a retrospective study, most of the patients in the study were not vaccinated and dynamic observation variation in antibody tires with the outcomes studied in a single patient. Second, there are limited patients in severe and critical patients compared to mild and moderate which may lead to biases in the results.

CONCLUSION

Serology (IgM and IgG) levels are high in RT-PCR positive group compared to clinical COVID-19. However, serology cannot be useful for the prediction of disease outcomes. The study also highlights the importance of doing serology at a particular time as antibody titers vary with the duration of the disease. In week intervals there was a significant correlation between clinical outcomes and serology on week 3.

ARTICLE HIGHLIGHTS

Research background

Predicting the severity of inflammatory response helps in managing critical patients using serology tests immunoglobulin G (IgG) and immunoglobulin M (IgM).

Research motivation

The importance of doing coronavirus disease (COVID) serology at a particular time as antibody titers may vary with the duration of the disease.

Research objectives

The objectivity was the correlation of the serology (IgM and IgG) with reverse transcriptase polymerase chain reaction (RT-PCR) status, disease severity (mild to critical), intensive care unit (ICU) admission, septic shock, acute kidney injury, and in-hospital mortality.

Research methods

This was a longitudinal study to correlate serum SARS-CoV-2 IgM and IgG serology with clinical outcomes in COVID-19 patients. We analyzed patient data from March to December 2020 for those who were admitted at All India Institute of Medical Sciences Rishikesh. Clinical and laboratory data of these patients were collected from the e-hospital portal and analyzed. A correlation was seen with clinical outcomes and was assessed using SPSS software.

Research results

Out of 494 patients, the mean age of patients was 48.95 ± 16.40 years and there were more male patients in the study (66.0%). The patients were classified as mild-moderate 328 (67.1%), severe 131 (26.8%), and critical 30 (6.1%). The mean duration from symptom onset to serology testing was 19.87 ± 30.53 d. In-hospital mortality was observed in 25.1% of patients. The seropositivity rate (*i.e.*, either IgG or IgM > 10 AU) was 50%. IgM levels (AU/mL) (W = 33428.000, $P \leq 0.001$) and IgG levels (AU/mL) (W = 39256.500, $P \leq 0.001$), with the median IgM/IgG levels (AU/mL), were highest in the RT-PCR-Positive group compared to RT-PCR-Negative clinical COVID-19. There was no significant difference between the two groups in terms of all other clinical outcomes (disease severity, septic shock, ICU admission, mechanical ventilation, and mortality).

Research conclusions

The study showed that serology levels are high in RT-PCR positive group compared to clinical COVID-19. The study also highlights the importance of doing serology at a particular time as antibody titers vary with the duration of the disease.

Research perspectives

The serology cannot be useful for the prediction of disease outcomes. In week intervals there is a significant correlation between clinical outcomes and serology on week 3.

FOOTNOTES

Author contributions: Panda PK, and Vivekanandhan V contributed to conceptualization; Panda PK contributed to methodology; Raina R contributed to software; Panda PK, Jain V, Suresh M, Omar BJ, and Kumar P contributed to validation; Raina R contributed to formal analysis; Saha S contributed to investigation; Panda PK contributed to resources; Suresh M contributed to data curation; Suresh M and Kumar P contributed to writing-original draft preparation; Panda PK and Omar BJ contributed to writing-review and editing; Panda PK contributed to visualization; Panda PK, Saha S, and Vivekanandhan V contributed to supervision; Panda PK, and Vivekanandhan V contributed to project administration; All authors have read and agreed to the published version of the manuscript.

Institutional review board statement: The Approval for this study was obtained from the institute ethics committee of All India Institute of Medical Sciences Rishikesh with approval no CTRI/2020/08/027169.

Informed consent statement: Consent is waived considering de-identification of the patient data.

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Data sharing statement: It will be made available to others as required upon requesting the corresponding author.

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