Eur. J. Oncol. - Vol. 22 - N. 3-4 - December 2017 | ISSN 1128-6598

EUROPEAN JOURNAL OF ONCOLOGY

The European Journal of Oncology is indexed by Excerpta Medica (EMBASE) and the Elsevier BioBASE

Mattioli 1885

Pubblicazione trimestrale - Poste Italiane s.p.a. - Sped. in A.P. - D.L. 353/2003 (conv. in L. 27/02/2004 n. 46) art. 1, comma 1, DCB Parma - Finito di stampare Marzo 2018

Ramazzini Institute

EUROPEAN JOURNAL OF ONCOLOGY

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Autorizzazione del Tribunale di Parma n° 14/97 del 11/6/1997
ISSN 1128-6598

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La testata fruisce dei Contributi Statali diretti di cui alla legge 7 agosto 1990, n. 250

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Pathophysiological roles, molecular interactions and clinical implications of long non-coding RNA CCAT2 in human cancer

Panagiotis Paliogiannis[1], Carlo Putzu[2*], Alessandro Giuseppe Fois[2], Pietro Pirina[2], Gianpaolo Vidili[2], Massimo Madonia[3], Diego Francesco Calvisi[2], Salvatore Sotgia[1], Ciriaco Carru[1], Angelo Zinellu[1]*

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Summary. Less than 2% of human genome has protein-coding ability, while over 90% is transcribed into non-coding RNA (ncRNA). NcRNA can be divided into small ncRNAs (<200 nucleotides) and long ncRNAs (>200 nucleotides). Small ncRNAs, including microRNAs (miRNAs), small interfering RNAs (siRNAs), and others have been investigated in recent years and recognized as key players in regulating cellular processes and diseases, including cancer. Long non-coding RNAs (lncRNAs) attracted increasing attention in recent years as researchers have revealed their crucial roles as regulators in embryogenesis, stem cell biology, and development. Furthermore, growing evidence indicates that dysregulation of lncRNAs is involved in cancer, and the regulatory functions and mechanisms of lncRNAs in human carcinomas have begun to emerge. Colon cancer-associated transcript 2 (CCAT2) is a lncRNA transcribed from human 8q24 gene desert, which has been recently found to be deregulated in several tumor types. In this review, we will briefly analyse the roles and mechanisms of lncRNAs CCAT2 involvement in human cancer, and we will discuss the future perspectives for research and clinical applications.

Key words: cancer, CCAT2, long non-coding RNA, carcinogenesis, oncogene, onco-suppressor

Introduction

The roughly 20.000 protein-coding genes represent nearly 2% of the human genome, while more than 90% comprises a non-coding portion that actively transcribes a massive and complex amount of RNA (1, 2). This part of the transcriptome was initially interpreted as transcriptional noise and called "dark matter" by some authors. Nevertheless, this very large amount of ncRNA was demonstrated to play numerous roles in normal cellular biology as well as in several pathological processes (3).

According to their size, ncRNAs are currently divided into two classes. The first class comprises small ncRNAs, as the recently discovered miRNAs, siRNAs and others, in addition to the well-known cellular RNAs (ribosomal, transfer, etc.). miRNAs are RNAs approximately 22 nucleotides long, which function as components of complex cellular networks involved in the regulation of numerous genes, generally by post-transcriptional silencing (4). The remaining class consists of long non-coding (lnc) RNAs, which are arbitrarily defined as non-coding RNAs greater than 200 nucleotides long (5). They have been discovered in 1990 by Brannan et al., and since then a considerable number has been described; several digital databases

* Contributed equally

provide information about the molecular and functional features of the currently known lncRNAs (6, 7). Generally, their length reaches 100 kilobases, without significant open reading frames (ORF); they are transcribed by RNA polymerase II or III, and can be located in the nucleus or cytoplasm (8, 9). Finally, their expression levels are usually lower than those of the protein-coding genes, and a certain tissue-specificity has been described (10-12).

LncRNAs are associated with different pre- and post-transcriptional functions, including nuclear architecture and import, immunity, imprinting, epigenetic regulations, cellular trafficking, splicing, precursors of smaller RNAs, and pluripotency of the embryonic stem cells (13). They can regulate gene expression at different levels, affecting cell proliferation, differentiation and apoptosis; therefore, they have been found involved in cancer development, maintenance, and progression (3, 14). Furthermore, they have been studied as risk, diagnostic, and prognostic markers as well as indicators of responses to treatments or recurrences.

CCAT2 in human cancer

Ling et al. reported in 2013 the discovery of a novel long ncRNA, CCAT2 (Colon Cancer Associated Transcript 2), which was found to be transcribed from the 8q24 genomic region (15). The CCAT2 genomic locus is highly conserved and harbours the SNP rs6983267; after its discovery, it was shown to be involved via various mechanisms in the pathogenesis and development of several human cancers (Table 1).

Table 1. Expression levels, interactions and clinical implications of CCAT2 in human cancer as depicted in the current scientific literature.

Cancer	Regul.	Interactions described	Clinical aspects	Reference
Breast	Up	ESR1, PGR, MYC, hormone status	Response to chemotherapy	Redis et al. (18)
Breast	Up	WNT signalling pathway	Prognostic marker	Cai et al. (19)
Breast	Up	p15	Prognostic marker, therapeutic target	Deng et al (20)
Breast	Up	TGF-β signalling pathway	-	Wu et al. (21)
NSCLC	Up	-	Marker of lymph node involvement.	Qui et al. (22)
NSCLC	Up	Pokemon, p21	-	Zhao et al. (23)
SCLC	Up	-	Prognostic marker	Chen et al. (25)
Esophageal	Up	MYC amplification	Prognostic marker	Zhang et al. (26)
Esophageal	Up	-	Diagnostic marker	Wang et al. (27)
Gastric	Up	EMT	Prognostic marker	Wang et al. (28)
Gastric	Up	-	Prognostic marker	Wu et al. (29)
Gastric	Up	-	Prognostic marker.	Wang et al. (30)
Colon	Up	WNT and MYC.	Relation with CIN score, putative markers	Ling et al. (15)
Colon	Up	BubR1	Prognostic marker	Kasagi et al. (32)
Colon	Up	mir145	Therapeutic target	Yu et al. (33)
Liver	Up	-	-	Zhou et al. (34)
Liver	Up	Snail2	Prognostic marker, therapeutic target	Xu et al. (35)
Glioma	Up	WNT signalling pathway	Putative diagnostic marker	Guo et al. (36)
Glioma	Up	EMT	Prognostic marker	Zeng et al (37)
Cervical	Up	-	Prognostic marker	Chen et al. (38)
Ovarian	Up	-	Prognostic marker	Huang et al. (39)
Cervical	Up	-	-	Wu et al. (40)
Prostate	Up	EMT	Prognostic marker	Zheng et al. (41)
Bladder	Up		Control by synthetic "tetracycline-on" switch system	Li et al. (42)

Breast cancer

Past studies have demonstrated that amplification of the 8q24 genomic region occurred more frequently in solid tubular or scirrhous mammary tumors than in less aggressive histotypes, and that a correlation exists between 8q24 DNA amplification profiles and breast cancer phenotype (16, 17). In other words, alterations in genes located on 8q24 are important for the development and/or progression of a consistent subgroup of primary breast cancers (BC), particularly those characterized by invasive behaviour. This led the team of investigators that discovered CCAT2 to further investigate its role and clinical correlations in breast cancer (18). For this purpose, the authors measured the mRNA levels of CCAT2 by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in a cohort of 26 non-neoplastic breast tissues and 30 breast cancer tissues, and evidenced significantly increased levels in tumor samples. Furthermore, in situ hybridization (ISH) showed a strong CCAT2 staining in epithelial cells of both neoplastic and non-neoplastic tissues; nevertheless, its expression was higher in the epithelial component of BC than in the corresponding component of healthy tissues. CCAT2 expression was detected in both invasive epithelial components and "in situ" epithelial lesions. However, in a distinct set of 15 unpaired normal breast tissues from another institute, CCAT2 expression did not vary significantly from the levels measured in 977 BC clinical specimens, despite it was significantly higher in the subgroup of tumors with aggressive pathological features (18).

As concerns the functional correlations, the authors found that increasing levels of ESR1 and PGR associated significantly with decreasing levels of CCAT2, while increasing levels of MYC (located on 8q24) were positively associated with CCAT2. Furthermore, a strong inverse association of CCAT2 expression with nodal status and hormone receptor status was demonstrated (18). Regarding prognosis, it was found that CCAT2 expression levels are informative only for patients with lymph node positive disease who have received adjuvant chemotherapy, and it was shown that it downregulates chemo-sensitivity to 5-fluorouracil in a rs6983267-independent manner (18).

In 2015, Cai et al. performed a study on human breast cancer specimens, cell lines and mice to better investigate the molecular interactions of CCAT2 and the WNT signalling pathway in breast cancer (19). The authors detected high expression levels of CCAT2 in breast cancer tissues and breast cancer cell lines, and evidenced that patients with high CCAT2 expression had a significantly poorer prognosis; the level of CCAT2 expression was correlated with overall survival rates. Experimental suppression of CCAT2 decreased cell proliferation and invasion *in vitro*, and inhibited tumorigenesis *in vivo*. The suppression of CCAT2 decreased the levels of β-catenin both in the cytoplasm and nucleus. CCAT2 knockdown reduced the expression of CCND1 and MYC in the BC cells employed, and evidenced a synergic effect of si-CCAT2 and FH535 (a WNT inhibitor) on WNT signalling activity (19).

Recent studies demonstrated that lncCCAT2 can also interact with EZH2 and inhibit the expression of p15 (20), and that its down regulation causes reduction of the protein expression levels of TGF-β, Smad2 and α-SMA in breast cancer cells (21). The pro-oncogenic role of CCAT2 in vivo was further confirmed in the latter studies.

Lung cancer

Levels of lncRNA CCAT2 in lung cancer were investigated by Qiu et al. (22). The authors examined paired non-small cell lung cancer (NSCLC) tissues and adjacent normal tissues (≥3 cm away from tumor) from 57 patients who received surgical resection for primary NSCLC and found that CCAT2 was significantly overexpressed in neoplastic tissues compared with normal tissues, with an average up-regulation fold of 7.5. It was also shown that over-expression of CCAT2 was significantly associated with the adenocarcinoma histotype. In addition, CCAT2 combined with CEA was found to be able to predict lymph node metastasis. The silencing of CCAT2 led to inhibition of proliferation and invasion in NSCLC cell lines, confirming the role of CCAT2 in promoting invasion. The authors concluded that CCAT2 is a lung adenocarcinoma-specific lncRNA providing aggressive neoplastic capabilities to NSCLC cells, which can represent a potential biomarker for lymph node metastasis (22).

The overexpression of CCAT2 in NSCLC tissues (neoplastic and adjacent non-neoplastic, 20 cases) and cell lines (Pc-9, H358, H1975 and HBE) was confirmed also in a recent article published by Zhao et al (23). Knock down of CCAT2 in NSCLC cells limited malignant growth and invasion, while artificial overexpression of CCAT2 led to opposite effects. In addition, CCAT2 knockdown significantly decreased the expression of POK erythroid myeloid ontogenic factor (Pokemon), and induced the expression of the p21 tumor suppressor. Furthermore, Pokemon overexpression could reverse the decrease of cell viability and cell invasion triggered by CCAT2 silencing (23). The authors, considering the numerous anti-neoplastic activities of p21 (24), claimed that the oncogenic potential of CCAT2 and Pokemon on NSCLC cells may depend on their inhibitory role over p21 (Figure 1).

CCAT2 was found to be overexpressed also in small cell lung cancer (SCLC) (25). Its expression was investigated in 15 pairs of primary SCLC samples, and pair-matched adjacent normal lung specimens. In addition, loss-of-function studies of CCAT2 in cell lines (DMS-53, H446 SCLC cell lines, and 16HBE normal bronchial epithelial cell line) were carried out. The results showed that CCAT2 expression is elevated in SCLC tissues and cell lines, and correlates with poor prognosis. Furthermore, knockdown of CCAT2 expression effectively suppressed SCLC cell growth and metastasis *in vitro*. These findings outline CCAT2 as an independent prognostic factor for SCLC patients, and a critical regulator of SCLC cell growth and metastasis (25).

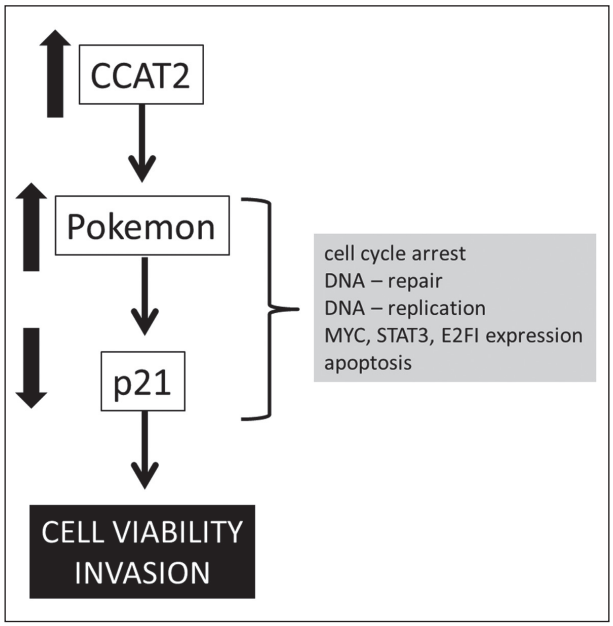

Figure 1. CCAT2 overexpression enhances the expression of Pokemon, which in turn supresses the expression of p21. The latter participates in the regulation of numerous biological processes, and its suppression leads to enhanced cell viability and neoplastic invasion.

Esophageal cancer

Two studies evaluating the expression levels and roles of lncRNA CCAT2 in esophageal cancer have been published to date. Zhang et al. evaluated CCAT2 levels in a series of surgically resected squamous cell esophageal cancer and matched para-cancerous tissues (26). They found that the tumours exhibit higher CCAT2 levels, positively correlated with advanced TNM stage, presence of lymphatic metastasis, and with the number of positive lymph nodes detected on pathological examination. An increased copy number of c-MYC correlated with increased levels of CCAT2, and the expression of CCAT2 in the c-MYC amplification group was significantly higher than that in c-MYC non-amplification group. The mean survival time was shorter in patients with high CCAT2 expression and c-MYC amplification; both CCAT2 expression and c-MYC amplification were established as independent prognostic factors by multivariate Cox regression analysis (26).

Similarly, in the study of Wang et al., CCAT2 was significantly overexpressed in squamous cell esophageal cancer tissues when compared with paired adjacent normal esophageal tissues, with an average fold of 7.18 (27). Furthermore, CCAT2 was mostly upregulated in KYSE410 cells, when normalized to a normal esophageal epithelium cell line (HEEC). A statistical correlation was found between CCAT2 expression levels and smoking status. Receiver operative curve (ROC) and the area under curve (AUC) were used to assess the diagnostic role of CCAT2, and it showed higher diagnostic performance than conventional serum biomarkers, such as alpha fetoprotein (AFP), CA153, and neuron-specific enolase (NSE).

Gastric cancer

Wang et al. investigated the biological behaviour and prognostic role of lncRNA CCAT2 in gastric cancer (GC) patients. The authors employed human gastric cancer and adjacent non-tumor tissues obtained from 85 surgical patients (28). The authors found that the expression levels of lncRNA CCAT2 were significantly higher in gastric cancer tissues than those in adjacent non-tumor tissues, and were closely correlated with higher incidence of lymph node and distant metastases. Moreover, patients with high CCAT2 expression had shorter overall survival and progression-free survival in multivariate analyses, indicating that it represents an independent factor for poor outcomes in gastric cancer patients (28). Similar results were reported also by Wu et al. in a recent study which confirms the role of CCAT2 in promoting cell proliferation and invasion in gastric cancer (29).

In a study performed in GC tissues and adjacent normal samples from 108 surgically treated individuals, as well as in normal epithelial (GES-1) and GC cell lines (SGC7901, MKN45, BGC-823 and MKN-28), CCAT2 was confirmed significantly upregulated in tumours and higher CCAT2 expression was correlated with poor survival (30). Furthermore, knockdown of CCAT2 inhibited cell migration, invasion and promoted epithelial-mesenchymal-transition (EMT) by downregulating E-cadherin expression and upregulating ZEB2, Vimentin and N-cadherin levels. Moreover, it was found that CCAT2 interacts with EZH2, LSD1, and H3k27me3, which in turn regulate E-cadherin and LATS2 expression (30). A similar mechanism of epigenetic regulation of specific genes from lncRNAs was described in an older article published by Tsai et al.; the lncRNA HOTAIR was found to serve as a scaffold for at least two distinct histone modification complexes (31). A 5′ domain of HOTAIR binds Polycomb Repressive Complex 2 (PRC2), while a 3′ domain binds to the LSD1/CoREST/REST complex, enabling the assembly of PRC2 and LSD1, and coordinating chromatin joined histone H3 lysine 27 methylation and lysine 4 demethylation (31). These studies indicate the complexity of the mechanisms of CCAT2–related oncogenesis in GC (Figure 2).

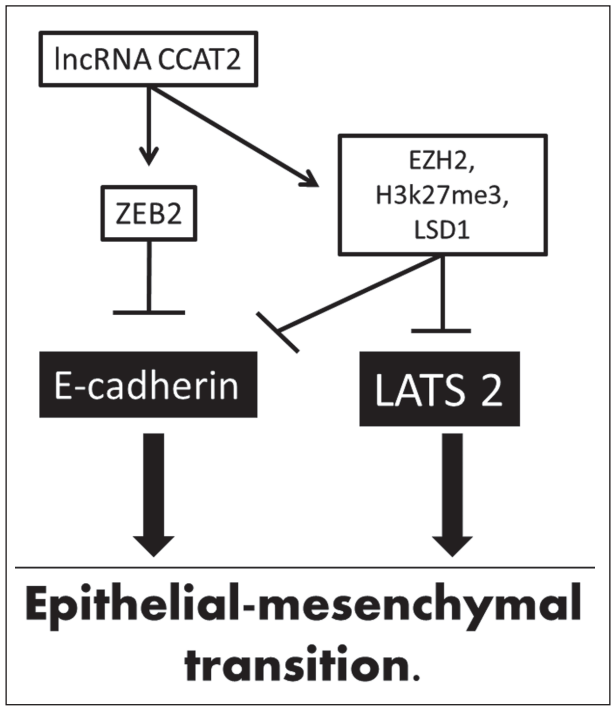

Figure 2. CCAT2 promotes epithelial-mesenchymal-transition (EMT) of gastric cancer cells by upregulating ZEB2 expression and repressing E-cadherin levels. Moreover, CCAT2 interacts with EZH2, H3k27me3, and LSD1, which negatively regulate E-cadherin and LATS2 expression, further promoting EMT.

Colorectal cancer

The discovery of lncRNA CCAT2 comes from experiments by Ling et al (15). The authors characterized CCAT2 and found that the newly identified transcript is expressed at much higher levels in colorectal tumor tissue than in the adjacent normal mucosa, and confirmed that the transcription occurs in the genomic sense orientation (15).

The authors described also an interesting model of CCAT2 locus involvement in CRC. In this model, a DNA loop brings the rs6983267 genomic region close to the c-MYC locus, and this physical association may contribute to the enhancer function of the SNP-containing region on c-MYC transcription. The enhancer region is transcribed into CCAT2, and the SNP status affects CCAT2 expression by an unknown mechanism. The CCAT2 transcript up-regulates WNT activity and increases expression of WNT

target genes (including c-MYC). This regulation by CCAT2 may lead to genomic instability and promote cell growth. Furthermore, the authors showed that c-MYC-regulated miR-17-5p and miR-20a participate in the CCAT2-enhanced cell invasion, and speculate that other mechanisms, such as c-MYC-related mechanisms or enhanced WNT signaling may coordinate the metastatic phenotype elicited by CCAT2. Finally, it was demonstrated that CCAT2 expression is regulated by the TCF7L2 transcriptional factor, indicating a positive feedback loop between CCAT2 and WNT signaling (15).

In a recent article, Kasagi et al. evaluated CCAT2 expression in 149 CRC patients and its associations with clinical and pathological characteristics, outcomes, rs6983267 genotypes, microsatellite status, DNA ploidy, and BubR1 expression (32). They confirmed that CCAT2 expression in cancer tissues was significantly greater than in healthy tissues, especially in metastases. The expression levels of the ncRNA and rs6983267 were not associated with the clinico-pathological features examined, they had not any prognostic significance, and cases with high CCAT2 expression were stable regarding microsatellite behaviour (32). Yu et al. observed that CCAT2 knockout negatively regulates the in vivo expression of miR-145 in colon cancer, impairing proliferation and differentiation. In contrast, stable up-regulation of CCAT2 decreased mature miR-145 (33). The authors also observed that CCAT2 is enriched in the nucleus and correlates with the expression of pre-miR-145 and hypothesized a novel pathogenic mechanism acting through selective block of miR-145 maturation by CCAT2 inhibition of pre-miR-145 export to cytoplasm.

Liver cancer

Zhou et al. and Xu et al. studied the involvement of CCAT2 in hepatocellular carcinoma (HCC). They observed that CCAT2 is upregulated in HCC tissues and human HCC cell lines (34, 35). Furthermore, they found that the overexpression of CCAT2 significantly promoted cell migration and proliferation, and inhibited apoptosis of HCC cells in vitro, confirming the same pattern observed in other malignancies; the suppression of CCAT2 expression resulted in opposing effects (34, 35). Furthermore, CCAT2 was shown once again to promote EMT and HCC progression by Snail2 induction (35).

Gliomas

To investigate the potential biological functions of CCAT2 in glioma, Guo et al. evaluated the CCAT2 mRNA expression in paired glioma tissues and adjacent normal tissues obtained from 134 patients with glioma. The authors found that the expression of CCAT2 is significantly higher in glioma tissues than in adjacent normal tissues, as well as in patients with advanced TNM stage in comparison to those with earlier stages (36). Additionally, the study of CCAT2 in U87-MG and U251 cells revealed that CCAT2 existed mainly in the nucleus of glioma cells. Both proliferation and colony formation assay revealed that silencing CCAT2 expression significantly inhibits its pro-neoplastic effects in cells. Moreover, knockdown of CCAT2 reduced tumor growth in nude mice. Also in this case, the authors showed that CCAT2 knockdown inhibits the transcriptional activity of WNT/β-catenin signalling pathway and the levels of downstream β-catenin target genes (c-MYC, MMP-7 and Cyclin D1), both at transcriptional and translational level (36). Thus, it is tempting to speculate that the inhibitory effect of decreased expression of CCAT2 on the malignant phenotype of glioma cells may be through a repression of the WNT/β-catenin signal pathway.

The results of Guo et al. regarding the expression levels and the impact of CCAT2 in glioma cell proliferation, migration and invasion were substantially confirmed by the findings of Zeng et al. (37). The authors studied also the interactions between CCAT2 and the expression of genes involved in the EMT in glioma cells. Of note, they found that suppressing lncRNA CCAT2 expression increased the expression of epithelial marker genes including E-cadherin and decreased the expression of mesenchymal marker genes including vimentin, N-cadherin, Twist, β-catenin, Snail. These findings suggest that knockdown of lncRNA CCAT2 could inhibit the EMT process. These results are consistent with those observed by Wang et al. in gastric cancer and those of other authors, depicting a combined mechanism which leads to EMT.

Gynecological malignancies

Regarding gynecological cancers, lncRNA CCAT2 has been studied in ovarian and cervical cancers. CCAT2 was found to be upregulated in both cancer types, and correlations between increasing expression levels and higher stages and metastasis were evidenced, as well as with poorer survival rates (38, 39). As in other types of cancer, knockdown of CCAT2 had an antineoplastic effect in cells of both ovarian and cervical cancers, in terms of cell proliferation, migration, invasion, and apoptosis (39, 40).

Urological malignancies

Zheng et al. employed human prostate cancer and paired adjacent healthy tissues and cell lines to study the implications of CCAT2 in prostate cancer onset and progression (41). The findings were very similar to those from other cancer types: the expression level of CCAT2 was higher in neoplastic tissues and cells compared to healthy tissues and normal WPMY- immortalized cells. Survival analysis revealed that patients with high CCAT2 expression had poorer overall and progression-free survivals than those with lower expression. Furthermore, multivariate analysis showed that the status of CCAT2 expression was an independent prognostic indicator for prostate cancer. Again, knockdown of CCAT2 could inhibit cell growth, migration, and invasion in vitro and stimulated EMT through abrogating N-cadherin, vimentin expression and intensifying the expression levels of E-cadherin, as observed in glioma cells (41).

CCAT2 was found to be upregulated also in bladder cancer, and its suppression caused a decrease of cell proliferation and migration as well as an induction of apoptosis in bladder cancer cells (42).

In summary, the results of the studies mentioned demonstrate the pro-oncogenic role of lncCCAT2 in numerous human cancers, both in vitro and in vivo. The main pathogenic networks involved include interactions with MYC, Pokemon, p21, p15, WNT, and other factors promoting the EMT. Therefore, lncCCAT2 may have several potential clinical applications as a biomarker of cancerogenesis and disease progression, as well as a pharmacological target and indicator of response to treatments.

CCAT2 as diagnostic and prognostic marker

CCAT2 was found to be an independent diagnostic marker in several malignancies. For instance, its value as a diagnostic marker was studied adequately and showed higher diagnostic performance than conventional serum biomarkers, like AFP, CA153, and NSE in esophageal cancer (27). Fan et al. performed recently a meta-analysis with the aim to clarify its functions as a prognosis marker in human malignancies (43). Six original studies were included with globally 725 cancer patients enrolled. The results outlined that high CCAT2 expression is significantly correlated with overall survival in cancer patients. A significant association was observed between high CCAT2 expression and poor progression-free survival in cancer patients. Furthermore, CCAT2 expression was significantly related to lymph node metastasis, distant metastasis, and tumor stage. The meta-analysis demonstrated that high CCAT2 expression may serve as a novel biomarker for poor prognosis and metastasis in cancers. Similar results were found in two further meta-analyses recently published (44,45).

Future perspectives

The discovery of CCAT2 and the mounting knowledge on its implications in human cancer represent a potential opportunity for translational applications. Nevertheless, a lot has to be done to this purpose. Firstly, it is necessary to confirm the oncogenic role of this lncRNA in other cancers not yet studied. Then, it is be useful to understand its clinical utility as s reliable diagnostic and prognostic marker. It is also necessary to determine if CCAT2 can be isolated from the blood of patients with cancer, to make its use as a disease marker even easier, with no need of tissue biopsies for determination. Moreover, it is worthy to better elucidate the functional interplay of CCAT2 with components of the WNT/b-catenin signalling pathway, p21, Pokemon, and the EMT process in order to better understand its role in cancer. Finally, it is crucial to explore the antineoplastic effectiveness of inhibitors of CCAT2 for the treatment of human malignancies.

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Received: 3.7.2017
Accepted: 19.12.2017
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EUR. J. ONCOL.; Vol. 22, n. 3-4, pp. 116-125, 2017

Distribution of *TERT* alternative splicing (AS) variants in pediatric brain tumors

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Summary. *Introduction:* The mechanism of telomerase regulation remains unclear, but has been suggested that *TERT* (telomerase reverse transcriptase) is regulated by alternative splicing (AS). Besides the full-length (FL) transcript, alternatively spliced variants have been described within the reverse transcriptase domain of *TERT* including, deletion alpha (α), beta deletion (β^-), and alpha beta deletions ($\alpha^-\beta^-$). Medulloblastoma (MB) and Ependymoma (EP) are two of more frequent brain tumors of childhood. We investigated and described the principal TERT transcripts; FL, α^-, β^- and $\alpha^-\beta^-$, and whether or not the presence of these patterns could be associated to clinical pathological characteristics and survival of pediatric EP e MB. *Methods:* We selected 58 MB and 43 EP samples. *TERT* AS variants were amplified by nested PCR (polymerase chain reaction) and the amplified products were electrophoresed on 2% agarose gel. *Results:* In general, around 5% of the samples of each group of tumors exhibited exclusively FL variant. *TERT* variants with deletion, exclusively or combined with others patterns, were detected in 70% of MB and 39% of EP tumors. 27% of MB and 60% EP did not show any of the patterns. We did not observed significant association between *TERT* splicing variants and clinical pathological characteristics of MB e EP tumors. *Discussion:* Since FL transcript is the only associated with reverse transcriptase activity, our results suggest that the association of *TERT* mRNA expression to clinical pathological characteristics of patients must be analyzed with caution. Further investigations will help to elucidate the complex mechanism involving AS of *TERT* gene and the function of deleted variants in tumorigenesis of pediatric brain tumors.

Key words: medulloblastoma, ependymoma, pediatric brain tumor, *TERT*, alternative splicing, therapeutic target

Introduction

Human telomerase is a ribonucleoprotein polymerase containing a protein catalytic subunit, the human telomerase reverse transcriptase (TERT), and an RNA component (TERC), that elongates telomeres by adding hexameric 5′-TTAGGG-3′ tandem repeats to the chromosomal ends (1, 2). The mechanism of regulation of telomerase remains unclear, but has been suggested that during development *TERT* is in part regulated by alternative splicing (AS) (3).

TERT gene on human chromosome 5p15.33 contains 16 exons can be spliced into multiple isoforms (3). To date, 22 isoforms of *TERT* have been identi-

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fied (4-6). Besides the full-length (FL) transcript with all 16 exons, none of the identified alternative spliced forms has reverse transcriptase activity and they cannot elongate telomeres (7, 8). The alternatively spliced variants within the reverse transcriptase domain of *TERT* include minus alpha (α^-), minus beta (β^-), or both minus alpha beta ($\alpha^-\beta^-$). These *TERT* splicing variants can lack reverse transcriptase function and their expression can modify telomerase activity levels (7-9). The inframe α^- deletion derived protein is a dominant negative inhibitor of telomerase activity, as would be expected if it forms heterodimers with the FL transcript-derived protein (8). The reading-frameshifting β^- deletion (182 bp) and $\alpha^-\beta^-$ deletion (218 bp) are believed to produce truncated proteins and may be subject to nonsense-mediated mRNA decay due to the premature stop codon (8, 10, 11).

Deletion in *TERT* variants are detected in a number of cancers and tumor cell lines and additionally during development, displaying expression patterns that reduce telomerase activity levels and may influence variations in telomere lengths (3-9). Several studies have been proposing *TERT* mRNA expression as an important prognostic factor with impact in the survival and clinical pathological characteristics of various neoplasias, including brain tumors. However, none of them identified the pattern of AS of *TERT* mRNA in pediatric brain tumors (12-21).

Between the pediatric brain tumors, Medulloblastoma (MB) is the most common embryonic neuroepithelial tumor of the cerebellum and added to other neuroectodermal tumors, accounts for 16-25% of cases. Approximately, one third of the cases remain incurable with negative impact in patients with higher long-term survival (12, 16). Of all primary tumors of the central nervous system in children, around 10% are Ependymoma (EP). This tumor arise from the ependymal lining of the ventricular system or the central canal of the spinal cord and its behavior is extremely variable, ranging from an aggressive course to prolonged survival with multiple relapses (16, 18). The clinical management of these tumors remains one of the more difficult in pediatric oncology (12, 16, 18).

Although several investigations of telomerase activity and/or expression in brain tumors of childhood have been made, to the best of our knowledge, this is the first study identifying the pattern of AS of *TERT* mRNA in pediatric brain tumors (12-27). Thus, we here aim to investigate and describe *TERT* transcripts, FL, α^-, β^- and $\alpha^-\beta^-$, and whether or not the presence of these isoforms could be associated to clinic pathological characteristics and survival of pediatric EP e MB.

Methodology

For the analysis of *TERT* transcripts, we selected a subgroup of 58 Medulloblastoma (MB) samples, and 43 Ependymoma (EP) samples. All samples used in this study were obtained from patients treated at the Pediatric Oncology Institute/Grupo de Apoio ao Adolescente e a Criança com Câncer - Federal University of São Paulo (IOP/GRAACC-UNIFESP). This was a retrospective study of samples collected sequentially between 2002 and 2013. Three cell lines (DAOY, SAOS, U2OS) were used as controls. Samples from each MB and EP were collected after informed consent was signed by patients/guardians according to the university's institutional review board (IRB/Federal University of São Paulo nº 333.158).

RT-PCR and nested PCR

TERT AS variants were amplified by nested PCR using primers designed according to GenBank, using Primer accession n°AF015950, based in previously published protocol (11, 28). The first round of amplification spanned a region that included all α^- and β^- deletion sites with forward primer 5'GCTGCTCAGGTCTTTCTTTTAT3' and reverse primer 5'GGAGGATCTTGTAGATGTTGGT3'. PCR was performed in 25 μL of reaction mixture using 1 μL of cDNA and 1U GoTaq polymerase (Promega, Madison, WI, USA) by incubation at 94°C for 2 minutes, followed by 25 amplification cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 90 seconds, and a final extension at 72°C for 5 minutes. This second round of PCR was carried out with 1 μL of the first-round PCR product, nested primer set and Taq. The nested primer set, forward 5'CCGCCTGAGCTGTACTTTGTC3' and reverse 5'CAGAGCAGCGTGGAGAGGAT3', produced four possible products, FL

(418 bp), α^- (382 bp), β^- (236 bp), and $\alpha^-\beta^-$ (200 bp). This round was performed by incubation at 94°C for 2 minutes, followed by 35 amplification cycles of 94°C for 20 seconds, 59°C for 20 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 2 minutes. Amplified products were electrophoresed on 2% agarose gel, stained with Gel Red (Biotium, Hayward, CA, USA), for size products identification.

Statistical analyses

Data analysis was performed using GraphPad Prism software, version 5 (San Diego, CA). Overall survival was defined as the time from diagnosis until the date of either the last follow-up or death. For the event free survival analysis, the duration was defined as the time from diagnosis until the occurrence of metastasis or local relapse. Overall survival and event-free survival curves were generated by applying the Kaplan-Meier method, and were then compared by the log rank test. Categorical data (age at diagnosis, gender, histological subtype, risk, and status for MB; age at diagnosis, gender, histological subtype, morphological classification, surgery extension, treatment, tumor location, and status for EP) and *TERT* AS patterns were studied using chi-square or Fisher exact tests. For this, different associations between the categorical variables, clinical-pathological characteristics and presence of the transcripts of the *TERT* variants, were tested. Statistical significance was taken as $p<0.05$. To provide level of confidence, we calculated the effect size and statistical power of tests, using R Core Team (2016) (URL http://www.R-project.org/).

Results

We analyzed 43 EP tumor samples and 58 MB tumor samples. A summary of the clinical pathological characteristics is demonstrated in Table 1. The complete data of the patients included in this study is in Tables 2 and 3. Statistical analysis are summarized in the Tables 4 and 5.

TERT AS variant patterns in MB tumors

For the 58 MB samples, 28 (48%) were considered high risk group, and 27 (46%) were low risk group.

Table 1. Clinical pathological characteristics of MB and EP tumor samples.

Medulloblastoma (MB)			Ependymoma (EP)		
	N	%		N	%
Total number of samples	58	100	Total number of samples	43	100
Risk			Location		
HR	28	48	*PF*	28	65
LR	27	46	*ST*	10	23
Status			*IM*	5	12
Alive	26	45	Status		
Dead	31	53	*Alive*	21	49
Histology			*Dead*	21	49
classic	45	77	Histology		
desmoplasic	3	5	*GI and GII*	33	77
anaplasic/large cells	3	5	*GIII*	10	23
nodular	7	12			
TERT transcript patterns			*TERT* transcript patterns		
NE	16	28	*NE*	26	60
FL	3	5	*FL*	2	5
FL + $\alpha^-/\beta^-/\alpha^-\beta^-$	38	65	*FL* + $\alpha^-/\beta^-/\alpha^-\beta^-$	15	35
$\alpha^-/\beta^-/\alpha^-\beta^-$	7	12	$\alpha^-/\beta^-/\alpha^-\beta^-$	8	18

IM= Intramedular, PF= Posterior fossa, ST= Supratentorial, PR= Partial resection, TR= Total resection, LR= Low risk, HR= High risk, GI= grade I, GII= grade II, GIII= grade III, NE= No mRNA expression, α^-= variant with deletion minus alpha, β^-= variant with deletion minus beta, $\alpha^-\beta^-$= variant with deletion minus alpha both minus alpha beta

Table 2. Clinical pathological characteristics and *TERT* alternative splicing variant patterns data of MB patients.

Patient	Age at diagnosis (years)	Histology	Risk	Staging	Overall Survival	Status	FL/α$^-$/β$^-$/α$^-$β$^-$
1	7,1	Classic	LR	R0M0	70,17	Dead	NE
2	13	Classic	HR	R+M0	12,73	Dead	FL/α$^-$/β$^-$
3	4	Desmoplasic	LR	R0M0	148,73	Alive	FL/α$^-$/β$^-$/α$^-$β$^-$
4	11	Classic	HR	R+M2	9,40	Dead	NE
5	13	Classic	LR	R0M0	28,10	Dead	FL/α$^-$/β$^-$/α$^-$β$^-$
6	2,7	NI	NI	NI	1,87	NI	NE
7	7	Classic	LR	R0M0	152,33	Alive	FL/α$^-$/β$^-$
8	1,6	Classic	HR	R+M+	3,53	Dead	FL/α$^-$/β$^-$/α$^-$β$^-$
9	9	Classic	HR	R0M+	0,77	Dead	NE
10	6	Classic	LR	R0M0	151,80	Alive	FL/α$^-$/β$^-$
11	18	Classic	NI	NI	7,63	Dead	FL/α$^-$/β$^-$/α$^-$β$^-$
12	7	Classic	HR	R+M+	57,87	Dead	FL/α$^-$/β$^-$/α$^-$β$^-$
13	11	Classic	LR	R0M0	11,60	Dead	β$^-$
14	5	Classic	HR	R+M+	5,40	Dead	NE
15	13	Anaplasic/Large Cells	LR	R0M0	16,77	Dead	NE
16	3, 4	Classic	HR	R+M+	21,93	Dead	NE
17	15	Classic	LR	R0M0	14,43	Dead	FL/α$^-$/β$^-$
18	2,11	Classic	HR	R+M+	3,07	Dead	FL/α$^-$/β$^-$/α$^-$β$^-$
19	16	Classic	LR	R0M0	64,90	Dead	β$^-$
20	1,6	Extensive nodularity	HR	R+M+	1,23	Dead	β$^-$
21	5	Extensive nodularity	HR	R0M+	123,47	Alive	NE
22	8	Anaplasic/Large Cells	LR	R0M0	11,17	Dead	FL/α$^-$/β$^-$
23	7	Large Cells	LR	R0M0	97,00	Dead	FL/α$^-$/β$^-$
24	15	Classic	LR	R0M0	6,57	Dead	FL
25	10	Classic	LR	R0M0	114,07	Alive	NE
26	6	Classic	LR	R0M0	113,10	Alive	FL/α$^-$/β$^-$/α$^-$β$^-$
27	14	Classic	HR	R+M0	40,27	Alive	FL
28	5	Classic	LR	R0M0	37,53	Dead	FL/α$^-$/β$^-$/α$^-$β$^-$
29	16	Classic	LR	R0M0	108,60	Alive	FL/α$^-$/β$^-$/α$^-$β$^-$
30	13	Classic	LR	R0M0	2,80	Dead	FL/β$^-$
31	8	Classic	LR	R0M0	104,53	Alive	FL/α$^-$/β$^-$/α$^-$β$^-$
32	9	Classic	LR	R0M0	102,03	Alive	FL/α$^-$/β$^-$/α$^-$β$^-$
33	1	Mixed (classic/desmoplasic)	HR	R0M0	3,97	Dead	NE
34	5	Classic	HR	R+M0	95,83	Alive	FL/β$^-$
35	7	Classic	HR	R+M0	98,53	Alive	FL/α$^-$/β$^-$/α$^-$β$^-$
36	7	Classic	LR	R0M0	93,83	Alive	FL
37	5	Classic	HR	R0M+	90,67	Alive	FL/α$^-$/β$^-$/α$^-$β$^-$
38	9	Classic	HR	R+M0	90,13	Alive	FL/α$^-$/β$^-$/α$^-$β$^-$
39	16	Classic	HR	R+M+	65,53	Dead	FL/α$^-$/β$^-$/α$^-$β$^-$
40	6	Classic	LR	R0M0	3,63	Dead	FL/β$^-$/α$^-$β$^-$
41	4	Classic	HR	R+M0	42,97	Dead	FL
42	2	Classic	HR	R0M+	37,80	Dead	FL/α$^-$/β$^-$
43	1	Extensive nodularity	HR	R+M0	5,13	Dead	FL/α$^-$/β$^-$
44	13	Classic	LR	R0M0	1370,43	Alive	NE
45	5	Classic	LR	R0M0	50,37	Alive	β$^-$
46	11	Classic	LR	R0M0	11,07	Dead	NE
47	0,3	Extensive nodularity	HR	R+M0	61,47	Alive	NE
48	5	Classic	LR	R0M0	45,60	Alive	NE
49	3,2	Desmoplasic	HR	R0M0	27,40	Dead	FL/α$^-$/β$^-$
50	3,3	Classic	HR	R0M0	37,67	Alive	α$^-$/β$^-$/α$^-$β$^-$
51	9	Classic	LR	R0M0	37,20	Alive	FL/α$^-$/β$^-$
52	4,5	Classic	LR	R0M0	35,60	Alive	NE
53	1,9	Extensive nodularity	HR	R0M0	30,60	Alive	NE
54	0,11	Extensive nodularity	HR	R0M0	32,00	Alive	FL/β$^-$
55	8,7	Classic	LR	R0M0	32,73	Alive	β$^-$
56	2,1	Extensive nodularity	HR	R+M0	11,07	Dead	FL/α$^-$/β$^-$

(continued)

Table 2 *(continued)*. Clinical pathological characteristics and *TERT* alternative splicing variant patterns data of MB patients.

Patient	Age at diagnosis (years)	Histology	Risk	Staging	Overall Survival	Status	FL/α^-/β^-/$\alpha^-\beta^-$
57	3,4	Classic	HR	R+M+	13,70	Alive	α^-/β^-/$\alpha^-\beta^-$
58	0,9	Classic	HR	R0M+	4,53	Dead	FL/α^-/β^-/$\alpha^-\beta^-$

LR= Low risk, HR= High risk, R0M0= no residual disease and no metastasis, R+M0= radiological residual disease alone, R0M+= presence of metastasis, R+M+= presence of residual disease and metastasis, NE= No mRNA expression, FL= Full Length, α^-= variant with deletion minus alpha, β^-= variant with deletion minus beta, $\alpha^-\beta^-$= variant with deletion minus alpha both minus alpha beta

Table 3: Clinical pathological characteristics and *TERT* alternative splicing variant patterns data of EP patients.

Patient	Gender	Age at diagnosis (years)	Diagnosis	Classification	Surgical extension	Recidive	Status	Overall Survival (months)	FL/α^-/β^-/$\alpha^-\beta^-$
1	F	1,4	IM	GII	PR	Yes	Dead	52,97	FL/β^-
2	F	1,9	PF	GII	TR	Yes	NI	107,90	β^-
3	M	17,2	PF	GII	PR	Yes	Dead	20,93	NE
4	M	2,7	PF	GII	TR	Yes	Dead	17,87	NE
5	F	8,8	ST	GII	TR	No	Alive	153,67	FL/α^-/β^-
6	M	0,8	ST	GII	NI	NI	Dead	0,47	FL/α^-/β^-/$\alpha^-\beta^-$
7	F	0,7	ST	GII	TR	No	Dead	12,30	NE
8	F	5,8	PF	GII	PR	Yes	NI	NI	β^-
9	M	5,1	PF	GII	TR	No	Alive	132,23	β^-
10	M	15,6	PF	GII	TR	No	Dead	124,67	FL
11	M	4,1	ST	GII	PR	Yes	Dead	31,57	NE
12	M	1,2	PF	GII	PR	Yes	Dead	94,83	NE
13	M	14,1	IM	GII	PR	No	Dead	114,70	NE
14	F	12,5	ST	GIII	TR	Yes	Dead	75,13	β^-
15	M	3,4	PF	GII	PR	Yes	Dead	18,03	β^-
16	M	16,1	ST	GIII	TR	Yes	Dead	70,80	NE
17	F	5,3	PF	GIII	TR	No	Alive	51,77	NE
18	F	9,8	PF	GII	TR	Yes	Dead	57,80	NE
19	M	0,10	PF	GIII	PR	Yes	Dead	98,23	FL/β^-
20	M	15,7	IM	GI	PR	No	Alive	100,73	NE
21	F	1,4	ST	GII	PR	No	Alive	90,47	NE
22	M	6,6	PF	GIII	PR	Yes	Dead	19,90	NE
23	M	NI	PF	GIII	NI	Yes	Alive	81,13	β^-
24	M	1,8	PF	GII	PR	Yes	Alive	100,37	NE
25	M	7,1	PF	GII	TR	No	Alive	28,97	FL
26	M	22	IM	GII	TR	No	Alive	62,30	β^-
27	F	0,4	ST	GII	PR	No	Alive	70,50	NE
28	M	13,6	PF	GII	TR	No	Alive	12,77	NE
29	M	1,3	PF	GII	PR	No	Alive	67,50	NE
30	F	6,8	ST	GIII	PR	No	Alive	66,13	NE
31	M	1,1	PF	GII	PR	Yes	Dead	28,43	NE
32	M	19	IM	GII	NI	NI	Alive	68,57	NE
33	M	17,1	PF	GII	TR	No	Alive	59,90	FL/α^-/β^-
34	F	10,2	PF	GIII	PR	Yes	Alive	72,57	NE
35	F	3,8	IM	GII	PR	Yes	Alive	55,47	NE
36	M	1,8	PF	GII	PR	No	Alive	58,90	NE
37	M	0,1	ST	GIII	PR	No	Alive	52,77	α^-/β^-/$\alpha^-\beta^-$
38	M	8	PF	GII	PR	No	Alive	48,80	NE
39	F	8,11	PF	GII	PR	NI	Dead	0,90	FL/$\alpha^-\beta^-$
40	M	1,1	PF	GII	PR	Yes	Dead	31,20	NE
41	M	4,6	PF	GII	TR	NI	Dead	20,23	NE
42	M	7,9	PF	GIII	TR	Yes	Dead	19,87	FL/α^-/β^-
43	M	11	PF	GII	TR	Yes	Dead	37,83	NE

IM= Intramedular, PF= Posterior fossa, ST= Supratentorial, PR= Partial resection, TR= Total resection, GI= grade I, GII= grade II, GIII= grade III, NI= No information, FL= Full Length, NE= No mRNA expression, α^-= variant with deletion minus alpha, β^-= variant with deletion minus beta, $\alpha^-\beta^-$= variant with deletion minus alpha both minus alpha beta.

Table 4. Expression of *TERT* transcripts according to clinical parameters of MB patients.

	NE		FL		FL+Variants		Variants		Total		p	DF	Effect Size	Power (%)
Histology														
Anaplasic/ Large Cells	1	6,7%	-	-	1	3,2%	-	-	2	3,5%				
Classic	10	66,7%	4	100,0%	24	77,4%	6	85,7%	44	77,2%				
Desmoplasic	-	-	-	-	2	6,5%	-	-	2	3,5%				
Extensive nodularity	3	20,0%	-	-	3	9,7%	1	14,3%	7	12,3%	0,5540	15	0,376955	0,3670 (36)
Large Cells	-	-	-	-	1	3,2%	-	-	1	1,8%				
Mixed (classic/ desmoplasic)	1	6,7%	-	-	-	-	-	-	1	1,8%				
Total	15	100,0%	4	100,0%	31	100,0%	7	100,0%	57	100,0%				
Risk														
HR	8	53,3%	2	50,0%	15	50,0%	3	42,9%	28	50,0%				
LR	7	46,7%	2	50,0%	15	50,0%	4	57,1%	28	50,0%	0,9134	3	0,061168	0,0625 (6)
Total	15	100,0%	4	100,0%	30	100,0%	7	100,0%	56	100,0%				
Status														
Alive	7	46,7%	2	50,0%	13	41,9%	4	57,1%	26	45,6%				
Dead	8	53,3%	2	50,0%	18	58,1%	3	42,9%	31	54,4%	0,7846	3	0,101035	0,0865 (8)
Total	15	100,0%	4	100,0%	31	100,0%	7	100,0%	57	100,0%				

NE= No expression, FL= Full Length, DF=degree of freedom, HR= High risk, LR= Low risk

Also, 26 (44%) patients are alive, 31 (53%) are dead, and 1 (2%) had no information. Of these 58 tumor samples, 45 (77%) were considered classic histology, 3 (5%) were desmoplasic histology, 3 (5%) were anaplasic/large cells and 7 (12%) were nodular histology.

In the group of MB samples, we observed the expression of at least one of *TERT* transcripts investigated in 41 (70%) of the 58 analyzed. In total, only 3/58 (5%) of samples exhibited exclusively FL variant. FL pattern combined with the presence of variants with deletion; inhibitory α^- deletion, nonfunctional β^- and $\alpha^-\beta^-$deletions were detected in 31/58 (53%) of the samples. 7/58 (12%) of the samples showed exclusively variants with deletion and 16/58 (27%) did not show any of the patterns (Figures 1 and 2). We did not observed significant association between *TERT* splicing variants and clinical pathological characteristics of MB patients (Table 4).

TERT AS variant patterns in EP tumors

Of 43 EP tumor samples, 28 (65%) were located at posterior fossa, 10 (23%) were supratentorial location, and 5 (11%) were intramedular. Among these patients, 21 (48%) are alive, 21 (48%) are dead, and 1 (2%) had no information. Of 43 tumor samples, 33 (76%) were considered grade I and II, and 10 (23%) were considered grade III. The treatment was based on chemotherapy for 24 (55%) patients and radiotherapy for 28 (65%) patients.

In the group of EP samples, we observed the expression of at least one of TERT transcripts investigated in 17 (39%) of the 43 analyzed. In total, only 2/43 (4%) of samples exhibited exclusively FL variant. FL pattern combined with the presence of the variants with deletion; inhibitory α^- deletion, nonfunctional β^- and $\alpha^-\beta^-$deletions were detected in 7/43 (16%) of the samples. 8/43 (19%) of the samples showed exclusively variants with deletion and 26/43 (60%) did not show any of the patterns (Figures 1 and 2). We did not observed significant association between *TERT* splicing variants and clinical pathological characteristics of EP patients (Table 5).

Table 5. Expression of *TERT* transcripts according to clinical parameters of EP patients.

	NE		FL		FL+Variants		Variants		Total		p	DF	Effect Size	Power (%)
Diagnosis														
IM	4	15,4%	-	-	1	14,3%	1	12,5%	6	14,0%				
PF	16	61,5%	2	100,0%	4	57,1%	5	62,5%	27	62,8%	>0,999	6	0,178759	0,1123 (11)
ST	6	23,1%	-	-	2	28,6%	2	25,0%	10	23,3%				
Total	26	100,0%	2	100,0%	7	100,0%	8	100,0%	43	100,0%				
Classification														
GI	1	3,8%	-	-	-	-	-	-	1	2,3%				
GII	20	76,9%	2	100,0%	5	71,4%	5	62,5%	32	74,4%	0,828	6	0,238308	0,1718 (17)
GIII	5	19,2%	-	-	2	28,6%	3	37,5%	10	23,3%				
Total	26	100,0%	2	100,0%	7	100,0%	8	100,0%	43	100,0%				
Surgical extension														
NI	1	3,8%	-	-	1	14,3%	1	12,5%	3	7,0%				
PR	17	65,4%	-	-	3	42,9%	3	37,5%	23	53,5%	0,267	6	0,381992	0,4219 (42)
TR	8	30,8%	2	100,0%	3	42,9%	4	50,0%	17	39,5%				
Total	26	100,0%	2	100,0%	7	100,0%	8	100,0%	43	100,0%				
Recidive														
No	11	45,8%	2	100,0%	2	40,0%	3	37,5%	18	46,2%				
Yes	13	54,2%	-	-	3	60,0%	5	62,5%	21	53,8%	0,611	3	0,260748	0,2455 (24)
Total	24	100,0%	2	100,0%	5	100,0%	8	100,0%	39	100,0%				
Status														
Alive	13	50,0%	1	50,0%	2	28,6%	4	66,7%	20	48,8%				
Dead	13	50,0%	1	50,0%	5	71,4%	2	33,3%	21	51,2%	0,642	3	0,216915	0,1865 (18)
Total	26	100,0%	2	100,0%	7	100,0%	6	100,0%	41	100,0%				

NE= No expression, FL= Full Length, DF=degree of freedom, IM= Intramedular, PF= Posterior fossa, ST= Supra tentorial, GI= Grade I, GII= Grade II, GIII= Grade III, NI=No information, PR= Partial resection, TR= Total resection

Discussion

In particular, numerous findings have been published on the prognostic value of *TERT* expression in pediatric MB and EP (14, 16-18, 22, 27). In many of these studies, *TERT* expression is present in 42% and 76% of the MB and EP samples, respectively, and has been proposed as a strong prognostic biomarker of poor survival. However, to the best of our knowledge, neither of these studies has taken into consideration the identification of *TERT* AS variant patterns (12, 15, 19-23, 25-27).

In our study, we observed *TERT* gene expression in 70% of MB and 39% of EP samples. The exclusive presence of FL form was detected in only 5% and 4% of MB and EP samples, respectively. FL transcript is the only one with reverse transcriptase activity and able to elongate telomeres. In a wide variety of telomerase-positive embryonic stem cells, adult proliferating stem cells, and cancer cells examined, only a small fraction of *TERT* transcripts are spliced into the FL form that generates the catalytically active protein (3, 29, 30). The need to fine-tune the regulation to produce "just the right amount" of telomerase may be because too little telomerase would not be enough to maintain telomere length leading to increased genomic instability in cancer cells, but too much telomerase may lead to runaway elongation of telomeres and result in adverse effects including growth inhibition of the cancer cells (31, 32).

In addition, we observed that *TERT* AS variants with deletions, α^-, β^- and $\alpha^-\beta$, exclusively or combined to FL form, were present in 53% and 16% of MB and

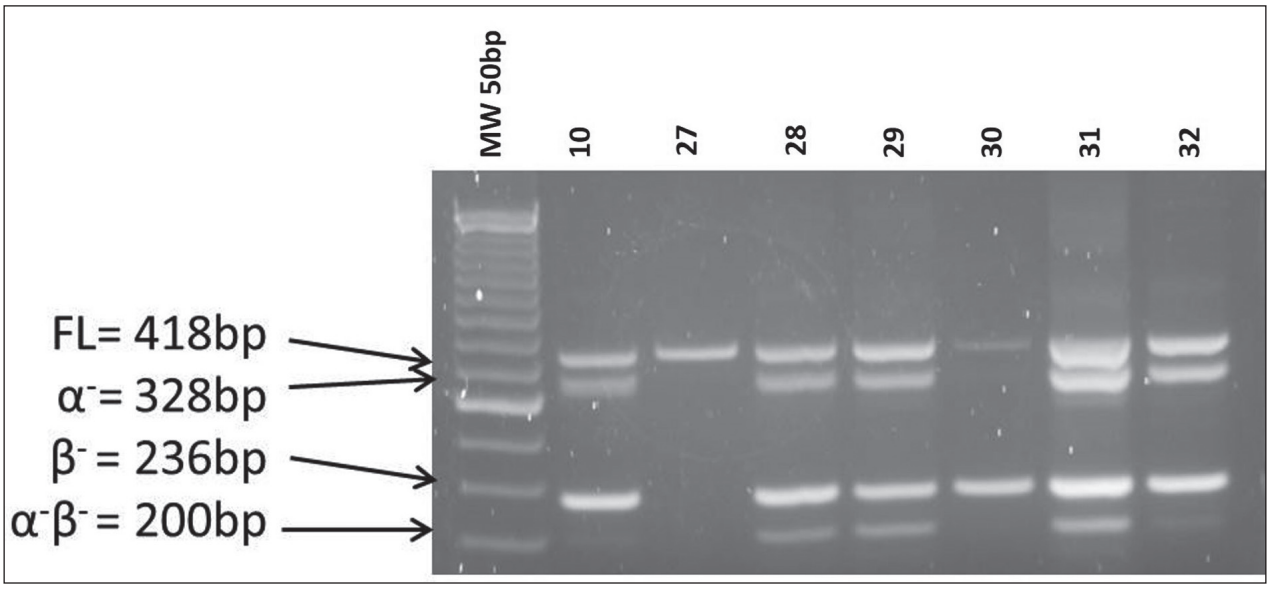

Figure 1. Identification of *TERT* transcripts in 2% agarose gel.

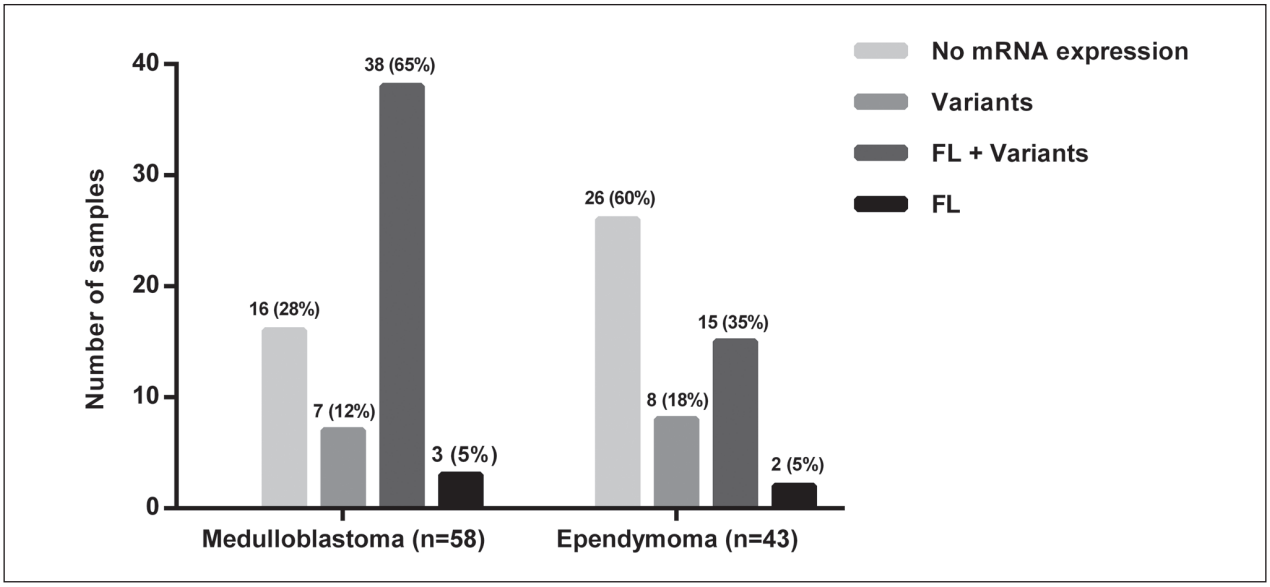

Figure 2. Distribution of *TERT* alternative splicing variant patterns in pediatric MB and. EP samples

EP tumors, respectively. We did not observed significant association between presence of *TERT* transcripts and the clinical pathological characteristics of these two groups of patients. In fact, the negative results observed are supported by the poor expressive power values found in each one of the statistical tests (Tables 4 and 5). The role of *TERT* variants in regulation of telomerase activity during tumorigenesis remains unclear and few studies have correlated *TERT* AS patterns in tumors with histopathological and clinical parameters (3, 29, 30, 32, 33). The use of different qualitative and quantitative methodologies to measure *TERT* mRNA in studies makes it difficult to directly compare interpretation of the results (7, 29, 34). Splicing variants of several proteins in tumor cells have been proposed as diagnostic or prognostic biomarkers and may provide

potential drug targets. The prospective use of more sensitive and refined methodologies, such as digital PCR, can collaborate to identify and quantify more precisely the splicing of low-abundance *TERT* transcripts (3, 29).

The establishment of associations between *TERT* AS variants and FL form and tumor clinical-biological behavior becomes even more difficult because of evidence that TERT protein has non-canonical functions that are unrelated to telomere lengthening. These in turn can be divided into the functions that still require the integrity of the catalytic site of *TERT* and the ones that do not (33). Among other functions, both, enzymatically active and inactive *TERT* modulate the Wnt pathway by acting as a transcription factor in beta-catenin complexes in positive and negative telomerase cells, indicating that this extratelomeric function is partially preserved in variant with deletion (24, 35, 36). Also, *TERT* protects normal and cancer cells from apoptosis independently of catalytic activity (37-39). Nevertheless, it is still unknown precisely the parts of *TERT* responsible for these effects and which specific variants retain these characteristics (3, 4, 7, 33, 38).

The presence of the FL form and the post-transcriptional processing of *TERT*, resulting in the variants with deletions as, α^-, β^- and $\alpha^-\beta^-$, could be a useful tool in predicting the progression of cancer. Future therapies, aimed at influencing the production of non-functional and/or dominant-negative variants, can be promising. Since FL pattern is the only associated with active telomerase enzyme, our results suggest that the association of *TERT* mRNA expression to clinic-pathological characteristics of patients, excluding the splicing alternative analysis, must be analyzed with caution. Further investigations will help to elucidate the complex mechanism involving AS of *TERT* gene and the function of variants with deletions in cancer maintenance, viability and progression, including the pediatric brain tumors.

Acknowledgements

This work was supported by awards from the FAPESP (The State of São Paulo Research Foundation: 2013/12281-4) and GRAACC (Grupo de Apoio ao Adolescente e Criança com Câncer).

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Received: 9.3.2017
Accepted: 14.12.2017
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EUR. J. ONCOL.; Vol. 22, n. 3-4, pp. 126-130, 2017

Identification and molecular characterization of a novel mutation in *MSH2* gene in a Lynch syndrome family

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Summary. *Background and aim of the work:* The Lynch Syndrome (LS) is associated with germline mutations in one of the MisMatch Repair (MMR) genes, including *MLH1, MSH2, MSH6, PMS2, MLH3* and *MSH3*. The molecular characterization of mutations in these MMR genes facilitates the pre-symptomatic diagnosis of subjects at risk to develop a colon cancer or a cancer LS-related. *Methods:* DHPLC and direct sequencing were performed for the mutation detection analysis. *Results:* In this study, we identified a novel frame shift mutation, the named is c.170delT in *MSH2* gene that determined a premature stop codon and consequently, the formation of a truncated protein (p. Val56Glyfs*7). This is a novel mutation, as it has not been reported before in the international scientific literature. This mutation was found in two subjects (father and son) belonging to a LS family. However, they showed a different phenotype disease. *Conclusion:* In this study, we identified and characterized a novel *MSH2* mutation; moreover, this study reaffirmed the importance of genetic testing in Lynch syndrome.

Key words: Lynch syndrome, HNPCC, *MSH2* gene, frame-shift mutation, novel variant *MSH2* gene

Introduction

Colorectal cancers are frequent causes of death in the world (1). Although most cases are sporadic, up to 5-6% develops in the context of gastrointestinal hereditary syndromes (2). Some syndromes are inherited in mendelian manner as they are due to alteration in a specific gene. These syndromes include familial adenomatous polyposis (FAP), hereditary non-polyposis colorectal cancer (HNPCC), PTEN hamartoma tumor syndrome (PHTS) and Peutz-Jeghers (3-7). With genetic testing for detection the mutation responsible of disease, it is possible to make differential diagnosis among these syndromes and to perform a pre-symptomatic diagnosis identifying the carriers of specific mutation in family at high colon cancer risk. Thus, DNA testing may change the individual's presumed risk status and affect decision-making by patients and their physicians regarding surveillance and management (8,9).

The HNPCC, also known as Lynch syndrome (LS) is inherited as dominant autosomal. The Lynch Syndrome is characterized by onset of colon cancer on average around 45 years as well as an increased risk of developing extra-colonic tumors such as endometrial cancer, ovarian, stomach, urinary and biliary tract (10,11). Identification of families affected by LS occurs by the Amsterdam Criteria (AC) and Bethesda guidelines (BG) (12, 13). LS is associated with mutations in MisMatch Repair (MMR) genes. Most of

mutations were found in the *MLH1* and *MSH2* genes that account for about 50% and 40% respectively of all mutations reported; only 10% of mutations were identified in the *MSH6* and 5% in *PMS2* (14, 15); a low percentage of mutations were identified in *MLH3* (16) gene and only one variant in *MSH3* gene was associated with LS phenotype (17). Mutations are distributed heterogeneously along each *MMR* gene, denoting the absence of "hot spot" mutations. The most frequently detected pathogenetic variants in *MLH1* and *MSH2* are small insertions/deletions or large genetic rearrangements (large deletions/insertions) that, at protein level, result in premature stop codon formation (18-20). Each mutation in one MMR genes is considered pathogenetic if determines the loss or malfunction of MMR complex. The loss of function of one MMR protein prevents to repair's complex to work properly and this determines a genetic instability known as microsatellite instability (MSI) at somatic level (21). At somatic level the MSI is detectable by immunohistochemistry (IHC) analysis (22).

In this study, we reported a novel frameshift mutations in *MSH2* gene that was identified in two affected subjects belonging to a family with Lynch syndrome.

Materials and methods

Patients

Our probands are a 20-year-old male (n. 1440) and his father with age of 49 years (n. 1439). The first developed a left colon cancer and two dysplastic adenoma at 19 years of age, while his father developed a left colon cancer at 44 years of age. The probands's family history was also positive for colorectal and extra-colonic cancer. A detailed pedigree is shown in Figure 1.

Furthermore, as negative controls we collected 100 healthy samples from Clinical Department of Laboratory Medicine of our Hospital (Federico II of Naples).

Sample from our patient was collected after being granted authorisation from our local Ethic Committee "Comitato etico per le attività Biomediche - Carlo Romano" of the University of Naples Federico II (protocol number 120/10). Once the authorisation has been obtained the study has received ethical approval, and participant' informed and written consent has been obtained.

Mutation analysis: Isolation of genomic DNA, amplification, dHPLC and sequencing

The genomic DNA was extracted from peripheral blood lymphocytes. Total genomic DNA was extracted from 4 mL peripheral blood lymphocytes using a BACC2 Nucleon Kit (Amersham Life Science).

All *MLH1* and *MSH2* exons were amplified, including intron-exon boundaries, on DNA extracted from blood lymphocytes of our patient, using customized primer sets (available on request). Prior to dHPLC analysis, the PCR products were run on an 1-2% agarose gel to check for unspecific amplicons. A Transgenomic Wave DNA Fragment Analysis System (3500 HT) was used to perform dHPLC analysis (Transgenomic Inc., Omaha, Nebraska, USA) using personal methods, available on request; subsequently, genomic DNA was re-amplified and sequenced in both the forward and reverse directions using an ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, Ca., USA).

Results

In this study, we have analyzed the DNA of our patients, n.1439 and n.1440. All *MLH1, MSH2,* exons were analysed by DHPLC; subsequently, we only sequenced exonic fragments that showed an abnormal chromatogram. In this manner, we identified a novel mutation in the *MSH2* gene that determined a nucleotide deletion (T) in position c.170. This mutation that is named c.170delT, determined a frameshift with premature stop codon and consequently, the formation of a truncated protein (p. Val56Glyfs*7). This mutation has not been reported before in the international database of INSIGHT-Group (20) and it was not detected in 100 healthy controls analyzed. Therefore, this mutation is considered as pathogenetic. No large rearrangements in *MLH*1 and *MSH2* genes were identified in our patients.

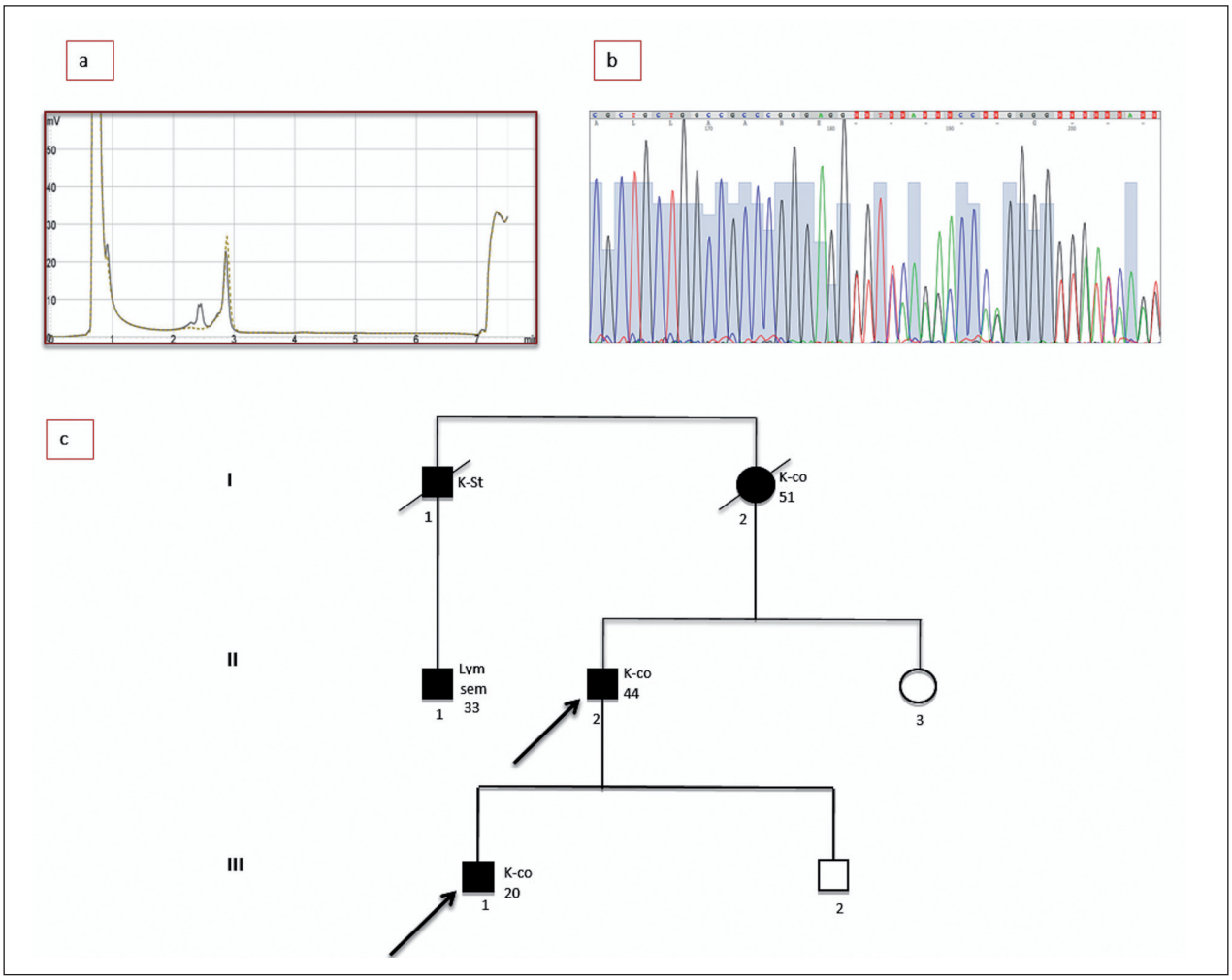

Figure 1. A. and **B.** Chromatogram and electropherogram showing the novel mutation identified in exon 1 of *MSH2* gene, c.170delT; **C.** Family pedigrees of our patients with a novel *MSH2* gene mutation. Symbols and abbreviations used are denoted as fellow: arrows, index case; black symbol, colorectal cancer or tumors associate with LS; Co, colon cancer; St, stomach cancer; Lym, lymphoma; Sem, seminoma. Number next to diagnosis denote age at onset

Discussions

In this study, we report the results of the detection mutation analysis of a family with Lynch Syndrome. The mutation identified, the c.170delT is a novel frameshift variant that causes the formation of a premature stop codon and hence of a truncated protein. Two subjects belonging to LS family were to carrier of this novel mutation in the *MSH2* gene. Both subjects showed a MSI-H status on DNA extracted from tumoral tissue embedded paraffin and loss of expression of MSH2 on tissue detected by immunohistochemistry analysis (data not shown). Therefore, the *MSH2* mutation c.170delT is, surely responsible of LS-phenotype in these patients and likely, in remaining affected subjects of family. These patients (father and son) were both affected by left colon cancer; however, the father, a 49-year-old patient developed an adenocarcinoma in left colon diagnosed at the age of 44 years and, the son developed an adenocarcinoma in left colon diagnosed at the age of 20 years. Hence, this novel mutation was associated with a phenomenon of generational anticipation in this family (23). So far, no clear correlation genotype-phenotype were described for LS families

(24), therefore, we could only speculate that modifier genes could be responsible of this generational anticipation (25). Moreover, this family's history was also positive for colorectal and extra-colonic cancer, Fig. 1. In particular, the cases of extracolic cancers were a lynphoma and a seminoma onset simultaneously, in a young relative of our probands (II-1, Fig. 1) and these two infrequent cancers in LS (11). Unfortunately, we were not able to performed genetic testing for this subject due to his limited availability.

In conclusion, this study remarks the phenotypic heterogeneity of LS and it enlarges the spectrum of MSH2 mutations. Moreover, this study reaffirms the importance to identify pathogenic mutations in LS families to facilitate pre-symptomatic diagnosis order to personalize the program of endoscopic surveillance for mutation carrier subjects, in particular in LS families whose the phenomenon of generational anticipation is present.

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Received: 3.2.2018
Accepted: 7.3.2018
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EUR. J. ONCOL.; Vol. 22, n. 3-4, pp. 131-137, 2017

Gemcitabine and vinorelbine: treatment option in recurrent platinum - resistant ovarian cancer

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Summary. *Background and aim of the work:* to evaluate the efficacy and toxicity of vinorelbine plus gemcitabine combination in patients who had recurrent platinum resistant ovarian cancer. *Patients and method:* twenty one patients with recurrent platinum resistant ovarian cancer were designated to receive vinorelbine 25 mg/m^2 plus gemcitabine 1 gm/m^2 on 1st and 8th day of each 21 – day cycle. *Results:* The Median age was 56 years (range 38-67). One patient (4.8%) achieved complete response and 5 patients (23.8%) had partial response with an overall response rate 28.57%. Median time to progression was 4 months and median overall survival was 11 months. Toxicity was modest and generally tolerated. Nine events (42.85%) of grade 3 hematological toxicity and only one event (4.57%) grade 3 non hematologic toxicity were observed. *Conclusion:* The combination of gemcitabine and vinorelbine is a moderately active regimen in recurrent platinum resistant ovarian cancer with an acceptable toxicity rating it as an option in treatment of such disease.

Key words: gemcitabine, vinorelbine, platinum resistant, recurrent ovarian cancer

Ovarian cancer (OC) is the fifth most frequently diagnosed malignant solid tumor in females and the leading cause of death among genital malignancies. Epithelial carcinoma constitutes about 85% to 90% of malignant ovarian tumors (1, 2).

In spite of recent evolution in cancer diagnosis, OC is commonly diagnosed in advanced stages and generally carries a dismal prognosis. Nevertheless, significant improvement in 5- year survival rates was reported in such patients treated with aggressive surgical cytoreduction and platinum- paclitaxel chemotherapy (3-5).

Although complete clinical response to first-line chemotherapy was achieved in 40%-60% of patients, relapse had been demonstrated in about 50% of these patients within 5 years (6, 7) and long-term remission was obtained in only 10%-15% of patients presenting with advanced stage OC (8). This high relapse rate is thought to be in part due to inherent or acquired resistance to chemotherapy (9-11).

Many factors were assumed to influence treatment strategies for recurrent OC including efficacy and toxicity of previous therapy, availability of drugs, financial support, patient compliance, and platinum sensitivity status being the most reliable factor commanding the treatment type (12, 13).

The length of disease free interval after platinum identified several categories of platinum sensitivity with different survival prognosis: platinum refractory defined as patients experiencing disease progression during or within one month after the end of therapy; platinum resistant disease as progression occurred through 6 months of therapy; and platinum sensitive as disease progression more than 6 months after the last platinum dose (14-16).

Currently, numerous platinum based chemotherapeutic regimens are available for treatment of recurrent platinum sensitive OC patients, and a greater chemotherapy regimens are also utilized as salvage treatment for recurrent platinum resistant OC patients, however

non of these regimens had been emerged as the standard therapy in this phase of the disease (17).

Gemcitabine is an active chemotherapeutic agent in treatment of OC. It has a convenient toxicity profile which suggest possible combinations with other active treatment (18-24). Vinorelbine is another active non platinum agent used in recurrent OC. Studies utilizing these agents reported response rates of 10%-30% in platinum resistant recurrent disease, and 30%-65% in platinum sensitive disease (25-29).

Giving the tolerability and convenience of administration of gemcitabine and vinorelbine and the only modest activity of each drug as a monotherapy in platinum resistant OC (25, 26), a combination of both drugs was tested to improve the dismal outcome of such disease (30-32).

This study was conducted to evaluate the efficacy and toxicity of vinorelbine plus gemcitabine combination in patients who had recurrent platinum resistant OC.

Patients and methods

This study was conducted in the Department of Clinical Oncology and Nuclear Medicine, Mansoura University Hospital, Egypt, on 21 patients.

Inclusion criteria

Patients had to have histologically proven epithelial OC recurred or progressed during or through 6 months of last injection of platinum based regimen. A maximum of 2 prior chemotherapy recurrence regimens were allowed. Other criteria include: age above 18 years, performance status ≤2 according to Eastern Cooperative Oncology Group (ECOG) status, adequate hematological, hepatic, and renal functions, and patient's informed consent.

Exclusion criteria

Previous or concurrent malignancies, underlying serious medical co-morbidities or uncontrolled infection, and patients received more than 2 different chemotherapy regimens for disease recurrence.

Treatment plan

Study patients were designated to receive vinorelbine 25 mg/m^2 over 10 minutes iv infusion followed by iv infusion of gemcitabine 1 gm/m^2 over 30 minutes. Both drugs were given on 1st and 8th day of each 21 – day cycle.

Treatment was planned for 2 cycles at least and response was assessed every 2 cycles. Patients expressed objective response (complete response, partial response or stable disease) proceeded on the treatment regimen for a minimum of 2 cycles after maximal tumor response, disease progression, unacceptable toxicity, or patient refusal.

Toxic effects were graded based on the National Cancer Institute Common Toxicity Criteria and chemotherapy was prohibited if any of these toxicities were detected: ≥grade 3 non hematologic toxicities, grad 3 neutropenia with fever ≥38.5° and/or infection, grade 3 thrombocytopenia with bleeding, grade 4 neutropenia, and grade 4 thrombocytopenia. Chemotherapy was delayed till toxic effects resolved to grade 1 or less. Dose reduction was not permissible. The use of erythropoietine and granulocyte colony stimulating factor (GCSF) was allowed as appropriate.

Patient assessment

Pretreatment assessment

Full history, clinical examination, complete blood count (CBC), biochemistry profile including CA125. The size and extent of the disease were documented by CT and/or MRI of chest, abdomen, and pelvis.

Assessment of response and toxicity

Before each treatment cycle, each patient underwent complete physical examination, CBC and biochemical profile. Response to treatment had to be assessed every 2 cycles by the same imaging technique used at base line and by examining CA 125 level however, progression could not be considered by CA 125 elevation alone.

Toxicity of treatment was graded on the basis of National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0

Survival

Time to disease progression (TTP) was considered as the time between 1st treatment infusion and the 1st detection of tumor progression. Overall survival (OS) was considered as the time period between the 1st day of treatment and date of last follow up or death.

Statistical analysis

Analysis of data was accomplished by SPSS program version 20. Demographic data was expressed by descriptive statistics. Analysis of TTP and OS was done using Kaplan-Meir method

Results

Patients characteristics

Between December 2009 and July 2015, 25 patients with platinum resistant recurrent OC were enrolled in this study. Four patients were excluded after initial inclusion: two patients lost to follow up after 1st injection, one patient had uncontrolled fever, and the last one developed elevated liver enzymes requiring dose reduction and exclusion from the study.

Base line characteristics of 21 evaluable patients were listed in table 1. Median age was 56 years. The serous cell type was the most common histological subtype (52%) with grade 2 differentiation in 57% of tumors. The majority of recurrences were abdominal and 7 patients had ≥3 sites of recurrence.

All patients were previously treated with paclitaxel and carboplatin regimen in the post operative adjuvant setting and 3 patients received it as neoadjuvant therapy.

Treatment administration

Three patients had 1ry platinum resistant tumors, only one of them received gemcitabine - vinorelbine after 1st tumor recurrence. Nine patients received gemcitabine - vinorelbine regimen on 2nd recurrence, 7 patients received it after their 3rd tumor recurrence, and 4 on 4th recurrence. Previous chemotherapy of study patients was listed in table 2.

Table 1. Baseline patients characteristics

Characteristics	N (%)
Age	
Median: 56	
Range: 38-67	
ECOG performance status	
0	5 (23.8)
1	13 (61.9)
2	3 (14.3)
Initial FIGO stage	
II	9 (42.85)
III	9 (42.85)
IV	3 (14.3)
Tumor histology	
Serous	11 (52.38)
Endometroid	5 (23.8)
Mucinous	3 (14.3)
Undifferantiated	2 (9.52)
Tumor grade	
Grade 1	1 (4.76)
Grade 2	12 (57.14)
Grade 3	8 (38.1)
Resistance to platinum	
1ry resistance	3 (14.3)
2ry resistance	16 (76.19)
2ry refractory	2 (9.52)
Site of recurrence	
Omental	5 (23.8)
Peritoneal	12 (57.14)
Lung	6 (28.57)
LN	6 (28.57)
Liver	4 (19.04)
Ascites	4 (19.04)
Pleural	2 (9.52)
Other	3 (14.3)
Involved sites	
One site	4 (19.04)
2 sites	9 (42.85)
≥3 sites	8 (38.09)

Gemcitabine - vinorelbine combination was given for a median of 6 cycles (range: 2-6). Treatment was delayed 1 week for 7 patients because of lack of hematological recovery. Hematological support was reported in 9 patients. No dose reduction was allowed.

Table 2. Details of chemotherapy

Chemotherapy	N (%)
Neoadjuvant chemotherapy	
Taxol/Carboplatin	3 (14.3)
Adjuvant chemotherapy	
Taxol/Carboplatin	21 (100)
1st recurrence chemotherapy	
Reinduction taxol/carboplarin	14 (65.75)
1st line	
Docetaxel/carboplatin	4 (19.04)
Docetaxel	2 (9.52)
Gemcitabin/Vinorelbin	1 (4.76)
2nd recurrence chemotherapy	
Reinduction taxol/carboplatin	4 (19.04)
1st line	
Docetaxel/Carboplatin	4 (19.04)
Docetaxel/Cisplatin	2 (9.52)
Docetaxel	2 (9.52)
Gemcitabin/Vinorelbin	2 (9.52)
2nd line	
Gemcitabin/Vinorelbin	4 (19.04)
Docetaxel	1 (4.76)
Paclitaxel	1 (4.76)
3rd recurrence chemotherapy	
1st line	
Docetaxel/Carboplatin	1 (4.76)
Docetaxel	3 (14.3)
2nd line	
Paclitaxel	4 (19.04)
Gemcitabin/Vinorelbin	4 (19.04)
3rd line	
Gemcitabin/Vinorelbin	2 (9.52)
4th recurrence chemotherapy	
2nd line	
Gemcitabin/Vinorelbin	4 (19.04)
3rd line	
Gemcitabin/Vinorelbin	4 (19.04)

Table 3. Response to treatment

Response	N (%)
Complete response	1 (4.76)
Partial response	5 (23.8)
Overall response	6 (28.57)
Stable disease	6 (28.57)
Progressive disease	9 (42.85)

Response

All 21 patients were evaluated for response (table 3). One patient achieved complete response (4.76%), 5 patients had partial response (23.8%), 6 patients (28.57%) had stable disease, and 9 patients (42.86%) had disease progression.

Survival

At time of final analysis, progressive disease had been reported in the entire patients, whereas 15 patients had died at different times over the study period.

Median TTP was 4 months (95% CI: 2.9-5.1 months) fig 1. Median OS was 11 months (95% CI: 6.5-15.5 months) fig 2.

Toxicity

Adverse events of treatment were listed in table 4. The study drugs were generally well tolerated with modest toxicity. Grade 3 neutropenia was observed in 5 patients (23.5%), 3 patients (14.4%) developed grade 3 anemia, and 1 patient had grade 3 thrombocytopenia. Except for 1 event of grade 3 diarrhea, mild to moderate (grade 1/2) non hematologic toxicities were reported including nausea and vomiting in 10 patients, constipation in 5 patients, phlebitis in 3 patients, fatigue in 8 patients, and peripheral neuropathy in 5 patients.

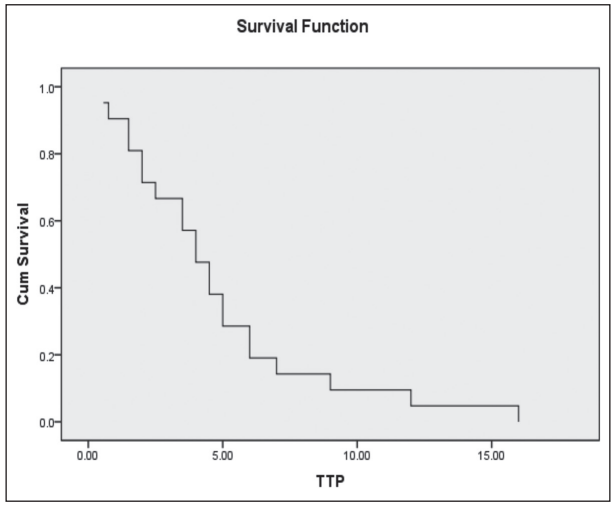

Figure 1. Time to progression of evaluable patients

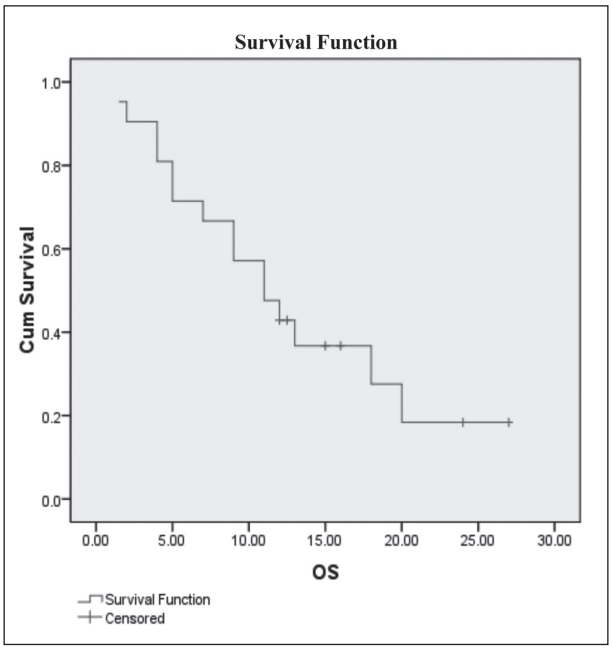

Figure 2. Overall survival of evaluable patients

Table 4. Toxicity of chemotherapy

Toxicity	G1/2 N (%)	G 3/4 N (%)
Hematological		
Anemia	12 (57.1)	3 (14.3)
Neutropenia	8 (38)	5 (23.8)
Thrombocytopenia	3 (14.3)	1 (4.76)
Non hematological		
Nausea	6 (28.6)	-
Vomiting	4 (19)	-
Diarrhea	1 (4.8)	1 (4.76)
Cramps	4 (19)	-
Constipation	5 (23.8)	-
Stomatitis	3 (14.3)	-
Phlebitis	3 (14)	-
Peripheral neuropathy	5 (23.8)	-
Fatigue	8 (38)	-

Discussion

Although OC is considered as one of the most chemo-sensitive tumors at initial diagnosis, tumor recurrence is expected in more than 80% of patients who had advanced disease with possible emergence of drug resistance. The principal chemotherapy regimens used for platinum sensitive recurrent OC are platinum based drugs. Conversely, in platinum resistant setting, several non platinum chemotherapy regimens are utilized. Gemcitabine and vinorelbine are non platinum chemotherapeutic agents with modest activity in OC.

In the present study, we evaluate both efficacy and safety of this doublet in treatment of recurrent platinum resistant OC patients.

Our study reported a response rate of 28.57% (one complete and 4 partial responses) and a disease control rate 52.38% . We also determined median TTP and median OS 4 months and 11 months respectively. These results were consistent with reports of different gemcitabine combination in recurrent platinum and taxane resistant ovarian cancer (31-34) and better than rates reported with either single gemctabine or single vinorelbine (24-26, 35). Chanpanitkitchot et al demonstrated lower response rate (5%) among platinum resistant patients who received gemcitabine alone or as combination therapy. They explained this by the poor inclusion criteria of their patients (36).

Data from clinical trials utilizing other combination chemotherapy in recurrent platinum resistant OC revealed similar moderate results. A combination of oral etoposide and intravenous irenotecan displayed an overall response rate 21.7%, TTP 4.1 months and OS 11.9 months (37). Mutch et al reported a non significant difference between gemcitabine and liposomal doxorubicin in response rates (6% vs 8% respectively), TTP (4 months vs 3 months respectively), and OS (13 months vs 14 months respectively) (12). The low response rates in this trial were due to the high proportion of platinum refractory patients.

Sehouli et al studied the advantage of adding either etoposide or gemcitabine to topotecan in treatment of recurrent ovarian cancer. They found no significant difference in response rates (36.3%, 36%, and 31.6%), TTP (7 months, 7.8 months, and 6.3 months) and OS (17.2 months, 17.8 months, 15.2 months) between treatment groups: topotecan monotherapy, topotecan plus etoposide, and topotecan plus gemcitabine (38). The high figures observed in this study were due to inclusion of patients with recurrent platinum sensitive disease.

Regarding the adverse events of gemcitabine – vinorelbine regimen used in this study, we illustrated a high tolerability and safety profile of this regimen, re-

flecting the acceptable and non overlapping toxicities of its individual drugs. We reported 9 events (43%) of hematological toxicities consistent with studies using gemcitabine combination (30, 32-34) and higher than the biweekly regimen used by Xenidis et al, because of its low dose threshold (31) and those reported with either drug (25, 26, 35, 39). Non hematologic toxicities were generally mild and rare which were also compatible with other reports (26, 30, 31, 34-36).

The outcome of traditional chemotherapeutic regimens in recurrent platinum resistant OC was still dismal. Response rates less than 30% had been reported. Novel strategies to overcome drug resistance and improve outcome have been emerged. The most currently studied in this phase are inhibitors of angiogenesis particularly bevacizumab. This is a humanized monoclonal antibody that attaches to vascular endothelial growth factor, inactivates it and thus blocks angiogenesis and inhibits tumor growth and metastases (40-42). Furthermore, the cytotoxic effect of chemotherapeutic agents is deemed to be enhanced by bevacizumab through normalizing tumor vascularity that improves oxygenation leading to better delivery and response to chemotherapy (43, 44).

Conclusion

The combination of gemcitabine and vinorelbine is a moderately active regimen in recurrent platinum resistant OC with an acceptable toxicity rating it as an option in treatment of such disease. However, further studies are needed for identification of ideal schedule and doses along with inclusion of potential active molecular targeted agents hoping to increase response rates, and improve both patients' survival and quality of life.

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Received: 29.12.2016
Accepted: 21.3.2018
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EUR. J. ONCOL.; Vol. 22, n. 3-4, pp. 138-149, 2017

Determination of anxiety, depression, and life satisfaction in lung cancer patients

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Summary. *Background and aim of the work:* This study aimed to determine the life satisfaction of patients with lung cancer by examining their levels of anxiety and depression. *Methods:* The study group consisted of 108 patients with lung cancer who were inpatients in a university hospital located in the Middle Black Sea region of Northern Turkey. The study was conducted between 28 March and 30 September 2013. Data were obtained using the Hospital Anxiety and Depression Scale (HADS) and Satisfaction with Life scale. *Results:* In this study, 3.7% of the patients were females, 96.3% were males, 96.3% were married, and 62.0% were primary school graduates. With regard to the disease stage, 40.7% were Stage 3. Among the group, 56.5% of the patients were receiving chemotherapy. In the patients, the treatment-related side effects were tiredness (78.7%), taste changes (68.5%), anorexia (65.7%), nausea (62.0%) vomiting (64.8%), and dyspnea (52.8%). According to the HADS scale, 97.2% of the patients had a risk of anxiety, and 100% had a risk of depression. The total score average of the patients on the Satisfaction with Life scale was 24.4±7.3. *Conclusions:* Almost all the patients had a risk of anxiety and depression. They were partially satisfied with their lives. They obtained relief from complementary and alternative treatments, such as biologically based therapies (herbs and dietary supplements) and mind-body control (prayer). The use of complementary and alternative treatments by lung cancer patients should be considered, as these may interfere with their prescribed treatment protocols.

Key words: anxiety, depression, life, lung, oncology, satisfaction, Turkey

Lung cancer is on the first rank for the males and on the second rank for the females among causes of the death depending on the cancer in 2012, with 1.6 million anticipated death ratio. Along with that, it is the leading for the females among causes of the death depending on the cancer by setting back the breast cancer in the developed countries (1). The highest cancer incidence ratio is seen in North America, Europe, Eastern Asia, and Uruguay in males, it is seen in North America, Europe, Australia, New Zealand, North Korea, and China in females (1).

Cancer frequency in Turkey shows similarities to the world and developing countries. The cancers that are mostly seen at males are lung cancer and prostate cancers (2). Lung cancer incidence in Turkey was 75.87/100 000 at males and it is 9.58/100 000 at females. Also anticipated annual case number in Turkey is calculated as 30239. Along with that more than 90% of the cases were male; it is notified that smoking has role at the etiology of the lung cancer (3). When phases of lung cancer in our country was reviewed, it was seen that 59.4% of them made far metastases and more than half of the patients in the lung cancer were diagnosed at advanced phase (2).

Compared to other cancer types, lung cancer has a high symptom load and a poor prognosis during and after the treatment (4), resulting in psychological stress (5) and psychological problems (4, 6). The aggressive treatment of lung cancer produces a number of side effects, which negatively affect both the treatment of

the disease and the patient's daily functioning (7). In patients with lung cancer, a major aim is to alleviate treatment-related side effects and improve the comfort and functioning of the patient (8).

In addition to the physical symptoms of the disease, the prognosis and treatment of cancer may produce strong emotions, such as anxiety and depression (9-11). As reported in the literature, when a patient receives a cancer diagnosis, a common response is anxiety and depression (11). Research has shown that cancer patients also commonly experience sadness at the time of the diagnosis and during their treatment (12). However, if these emotions persist and become permanent, they can affect their daily functioning (13).

Treatment-related side effects, including dyspnea, cough, and hemoptysis, can decrease the functional performance of the patient, resulting in increased anxiety levels (4, 14). Social isolation, fatigue, anorexia, weight loss, sleep disorders, cognitive disorders, decreased libido, and psychomotor retardation are well-known side effects of major depression (9, 12, 15). Although depression is very common among lung cancer patients (16), research suggests that the symptoms of depression may be overlooked in cancer patients (9, 12, 15).

A recent review of psychosocial aspects of lung cancer found that one-fourth of patients experienced depression and other psychosocial problems during the disease and that the risk was much higher among patients with small-cell lung cancer compared to patients with other types of cancers (6). Other studies of lung cancer patients found that the risk of depression was associated with the level of education, with those who had a low level of education having a higher risk, and that a psychiatric consultation was recommended for 8% of patients who had major or minor depression symptoms (17). Zabora et al. (2001) stated that one of every five patients with cancer suffered distress and that the prevalence was 43.4% in those with lung cancer (5). Hamer et al. (2009) stated that psychological distress increased lung cancer-related mortality (18).

A life-threatening disease, such as cancer, affects both life quality and life satisfaction (19). Life satisfaction refers to general satisfaction with life (20). It was stated in the literature that it is concept enough and appropriate for detecting the life satisfaction as relevant to the life quality regarding to the health (21, 22). The level of life satisfaction has a direct impact on the quality of life. Given the importance of life satisfaction, research has focused on determining the coping processes that people use in times of stress (23). As every individual assigns different meanings and value to health (24), the Satisfaction with Life scale developed by Diener et al. (1985) contains a subjective well-being component (24). According to the literature, this scale can be used to measure mental health and to predict future behavior. The Satisfaction with Life scale can be also be used to assess the subjective life quality of individuals with serious diseases whose anxiety is linked to their poor health (25).

Knowledge of the anxiety and depression levels of lung cancer patients and their level of life satisfaction can help to improve their quality of life. To achieve the goal of improving the life quality of lung cancer patients, there is a need for further studies that consider cross-country, cross-regional, and cross-cultural divergences in addition to relevant findings and data collected from such studies. In previous research the focus has mainly been on anxiety and depression levels (4, 16, 17, 26-37) among lung cancer patients, but no study has yet carried out a cultural interpretation of factors related to patients' anxiety and depression level, and life satisfaction. That missing link in the chain was the driving motive of the present study. Also to the best of our knowledge, this is the first study on treatment-related side effects in the Middle Black Sea region of Northern Turkey. The results of this study may be useful in terms of developing appropriate strategies for national cancer action plans devoted to preventing anxiety and depression among oncology patients and increasing their life satisfaction and quality of life. In addition, having awareness on the prevalent symptoms among lung cancer patients, and their self-care strategies will help healthcare professionals to provide optimum care and treatment options to their patients.

Research questions

To determine the anxiety, depression, and life satisfaction levels of inpatient patients with lung cancer, the following questions were posed:

- What are the most frequent treatment-related symptoms?
- What are the levels of anxiety and depression?
- What is their level of life satisfaction?
- Do socio-demographic characteristics and the clinical status of the patients affect their levels of anxiety, depression, and life satisfaction?
- Does culture play a role in the methods that patients use to relieve treatment- and disease-related symptoms?

Methods

Study design and sampling method

This descriptive study was conducted between 28 March and 30 September 2013 in a university hospital located in the Northern Turkey. Patients with a lung cancer diagnosis who were 18 years old and older, literate, willing to participate in the study, and were physically and mentally capable were notified about the research and asked to sign a participation consent form. In this research, an initial attempt was made to access the entire population, but ultimately non-volunteering patients, those who had not properly completed the questionnaire form, and those with a cognitive disorder (n=17) were excluded from the research; thus, the final sample comprised 108 lung cancer patients. The rate of questionnaire completion was 84.2%.

Data collection

In this research, data were collected via a 28-item questionnaire form developed by researchers in line with relevant literature (4, 17, 28-30, 34-36, 38) to determine patients' sociodemographic characteristics (age, gender, education level, occupation, marital status, socio-economic status, family type, number of children, employment status, health insurance status, inhabited settlements, perception of the prognosis of the disease, illness perception, dissatisfaction with the medical treatment received, presence of a chronic illness, treatment-related side effects and symptoms). Depression and anxiety were evaluated using the Hospital Anxiety and Depression Scale (HADS), and life satisfaction was evaluated using the Satisfaction with Life scale. Clinical data on the patients (diagnosis, stage of the disease, applied treatments, time of the diagnosis, etc.) were obtained from the patient's files.

The questionnaire form was pre-tested as a pilot among a group of 5 patients; patients participating in the pilot study were not included in the sample. The data were collected by the researchers after explaining the objectives of the study to the participants. All the patients were advised that their participation was entirely voluntary and anonymous (i.e., no names would be written on the survey forms). They were also informed that the data collected in the study would only be used within the scope of the study. The data collection took 10-15 min.

Data collection tools
HADS

The HADS was developed by Zigmond and Snaith (1983) with the aim of detecting the risk of anxiety and depression among patients and the severity of these symptoms (39). The validity of the scale in a Turkish population was confirmed by Aydemir et al. (1997) (40). This scale is not utilized to diagnose physically impaired patients or those applying to primary care health services, but it is employed to diagnose anxiety and depression rapidly to identify risk groups. Responses given on a 4-point Likert-type scale are scored with numbers 0-3. The HADS is easy to administer because it is short and easily understandable. The participants filled in the form on their own and marked the article that was most appropriate to their situation. Out of 14 questions in total, seven (odd numbers) measure anxiety and seven (even numbers) measure depression. For the anxiety subscale, the scores of items 1, 3, 5, 7, 9, 11, and 13 are added; for the depression subscale, scores of items 2, 4, 6, 8, 10, 12, and 14 are added. On both subscales, the lowest score that patients can receive is 0 and the highest is 21. At the end of validity test of this scale, the cutoff score of the HAD Scale's Turkish form was measured as 10 for the anxiety scale and 7 for the depression scale. Accordingly, those receiving more than 10 points from the anxiety subscale are considered to be at risk of anxiety, and those receiving more than 8 points on

the depression subscale are considered to be at risk of depression. In the reliability test of the Turkish form, Cronbach's alpha coefficient was computed as 0.85 for the anxiety subscale and measured as 0.77 for the depression subscale (40). In this study, the Cronbach's alpha reliability coefficient of the HADS was 0.71.

Satisfaction with Life scale

The Satisfaction with Life Scale (Diener et al., 1985) was developed with the aim of scaling the life satisfaction (24). This scale was adapted to a Turkish population by Köker (1991) (41). It is a Likert type scale consisting of 5 items, to which answers range from "completely opposing" to "absolutely accepting" with 1 to 7 points. The total score varies from 1-35. Scores of 31-35 denote a high level of satisfaction with life. Scores of 26-30 denote satisfied, and scores of 21-25 signify partially satisfied. A score of 20 denotes a medium level of satisfaction, whereas scores of 15-19, 10-14, and 5-9 denote some dissatisfaction, a medium level of dissatisfaction, and extreme dissatisfaction, respectively. The Cronbach's alpha reliability coefficient of the Satisfaction with Life scale was 0.92.

Statistical Analysis

Statistical analysis of the data was done using SPSS 15.0. Percentages, one-way ANOVA and the student's *t*-test were used to analyze the data.

Results

One hundred-eight lung cancer patients took part in the study. As shown in Table 1, 3.7% of the patients were females, and 96.3% were males. Of these, 96.3% were married. In the study group, 62.0% had graduated from primary school, 12.0% had graduated from secondary school, 5.6% had graduated from high school, and 5.6% had graduated from university graduated. In this study, 95.4% had social security, 14.8% were working, 37.0% were retired, 30.6% were farmers, 3.7% were housewives, and 1.9% were civil servants. The incomes of 52.8% of the patients were less than their expenditures. In terms of their distribution, 28.7% lived in the province, 38.0% lived in the district, and 33.3% lived in villages. With regard to the family structure, 68.5% had a nuclear family structure. The average age of the patients was 61.9±8.9 (Table 1).

Table 1. Socio-Demographic characteristics of the patients (N=108)

Characteristics	M	(SD)
Mean age	61.9	(8.9)
	n	**(%)**
Gender		
Female	4	(3.7)
Male	104	(96.3)
Marital status		
Married	104	(96.3)
Single/widow	4	(3.7)
Educational level		
Literate	16	(14.8)
Elementary	67	(62.0)
Intermediate school	13	(12.0)
High school	6	(5.6)
University	6	(5.6)
Social insurance		
Present	103	(95.4)
Absent	5	(4.6)
Employment status		
Working	16	(14.8)
Nonworking	92	(85.2)
Job		
Civil servant	2	(1.9)
Employee	4	(3.7)
Retired	40	(37.0)
Self-employed	25	(23.1)
Farmer	33	(30.6)
Housewife	4	(3.7)
Income status		
Income less than expenditure	57	(52.8)
Income equal to expenditure	40	(37.0)
Income greater than expenditure	11	(10.2)
Place of residence		
City	31	(28.7)
Town	41	(38.0)
Village	36	(33.3)
Family type		
Large	34	(31.5)
Nuclear	74	(68.5)

In this study, 84.3% of the patients had been diagnosed with cancer in the previous 0-6 months, and 40.7% had Stage 3 disease, 56.5% were receiving chemotherapy. The following symptoms were reported: tiredness (78.7%), taste changes (68.5%), anorexia (65.7%), vomiting (64.8%), nausea (62.0%), dyspnea (52.8%), alopecia (47.2%), pain (45.4%), and weight loss (39.8%). 52.8% perceived the prognosis of disease to be at a medium level. In the study, the following diseases were present: chronic disease (38.9%), atherosclerosis (38.9%), cardiac disease (13.9%), diabetes (7.4%), and hypertension (18.5%). A total of 90.7% of the study group was satisfied with their treatment, and 63.3% defined their level of satisfaction as medium. A total of 69.4% of the patients obtained a benefit from herbal treatments. Others achieved symptom relief from diet (44.4%) and prayer (79.6%). In the group, 88.9% of smokers quitted smoking after the disease was diagnosed (Table 2).

Table 3 presents the HADS score averages. The average HADS-A score was 18.5±2.7, and the average HADS-D score was 16.5±2.3. According to the HADS-A dimension of the scale, 2.8% of the patients were under the cutoff score (0-10 points), and 97.2% were over the cutoff score (11-21 points). With regards to the HADS-D, 100% of the patients were over the cutoff score (8-21 points) (Table 3). There was no association between the HADS-A scale score averages and the patients' socio-demographic and clinical statuses. However, on the HADS-D, there was a statistically significant association between the score averages and age (F=2.985 p=0.035), income level (F=4.146 p=0.018), social security status (t=2.279 p=0.025), martial status (t=2.029 p=0.045), and sex (t=3.449 p=0.001). The HADS-D score was higher among those aged 64-76 and among those whose incomes were higher than their expenditures. It was also higher among those who had social security and who were married and male.

The total score average on the Satisfaction with Life scale was 24.4±7.3, and most of the patients were partially satisfied with their lives (Table 4). There was a statistically significant difference between the groups in terms of age (F=5.485 p=0.002), income level (F=5.976 p=0.003), sex (t=3.449 p=0.001), marital status (t=2.029 p=0.045), and social security status (t=2.279 p=0.025). The Satisfaction with Life scale score was higher among those aged 64–76 and among those whose incomes were higher than their expenditures. It was also higher among those who had social security and who were married and male.

Table 2. Clinical characteristics of the patients (N=108)

Characteristics	n	(%)
Time since diagnosis (month)		
0–6 days	91	(84.3)
7–13 days	17	(15.7)
Phase of the disease		
Nonsmall cell stage 1	4	(3.7)
Nonsmall stage 2	8	(7.4)
Nonsmall cell stage 3	44	(40.7)
Nonsmall cell stage 4	29	(26.9)
Smallcell limited stage	9	(8.3)
Smallcell wide stage	14	(13.0)
[a]**Applied treatments**		
Chemotherapy	61	(56.5)
Radiotherapy	7	(6.5)
Chemotherapy + radiotherapy	37	(34.3)
Surgical treatment	11	(10.2)

(continued)

Table 2 ***(continued)*****.** Clinical characteristics of the patients (N=108)

Characteristics	n	(%)
[a] **Symptom-related side effects of the treatment**		
Ache	49	(45.4)
Anorexia	71	(65.7)
Weight loss	43	(39.8)
Taste changes	74	(68.5)
Alopecia	51	(47.2)
Nausea	67	(62.0)
Vomiting	70	(64.8)
Fatigue	85	(78.7)
Dyspnea	57	(52.8)
Constipation	40	(37.0)
Perceived disease prognosis		
Good	42	(38.9)
Medium	57	(52.8)
Bad	9	(8.3)
Presence of absence of a chronic disease		
Yes	42	(38.9)
No	66	(61.1)
[a]**Chronic diseases (*n*=42)**		
Cardiac disease	15	(13.9)
Diabetes	8	(7.4)
High blood pressure	20	(18.5)
Atherosclerosis	42	(38.9)
Satisfaction with the received medical treatment		
Yes	98	(90.7)
No	10	(9.3)
If yes, level of satisfaction(*n*=98)		
Low	4	(4.1)
Medium	62	(63.3)
High	32	(32.6)
[a]**What thing/things do you do to alleviate the symptoms?**		
Herbal treatment	75	(69.4)
Regulating diet	48	(44.4)
Praying	86	(79.6)
Nothing	28	(25.9)
Smoking history		
Quit smokers	96	(88.9)
Never smokers	12	(11.1)

[a]More than one answer was given

Table 3. Average scores on the HADS-A and HADS-D subscales

Scores	Score range	n	%	Mean ± SD	Total score average
HADS-A scores	0-10 points	3	2.8	9.66±0.57	18.5±2.7
	11-21 points	105	97.2	18.74±2.25	
HADS-D scores	0-7 points	-	-	-	16.5±2.3
	8-21 points	108	100.0	16.55±2.26	

Table 4. Distribution of the patients' scores on the Satisfaction with Life Scale (N=108)

Items	Strongly disagree *n* (%)	Disagree *n* (%)	Slightly disagree *n* (%)	Neither agree nor disagree *n* (%)	Slightly agree *n* (%)	Agree *n* (%)	Strongly agree *n* (%)	Mean ±SD
In most ways, my life is close to ideal	5 (4.6)	8(7.4)	6 (5.6)	16 (14.8)	21 (19.4)	35 (32.4)	17 (15.8)	5.0±1.7
The conditions of my life are excellent	5 (4.6)	9 (8.3)	8 (7.4)	24 (22.2)	22(20.4)	30 (27.8)	10 (9.3)	4.6±1.6
I am satisfied with my life	5 (4.6)	6 (5.6)	5 (4.6)	13 (12.0)	26 (24.1)	32 (29.6)	21 (19.5)	5.1±1.6
So far, I have achieved the things I wanted to in life	6 (5.6)	8 (7.4)	9 (8.3)	16 (14.8)	21(19.4)	29 (26.9)	19 (17.6)	4.9±1.7
If I could live my life over, I would change almost nothing	10 (9.3)	8 (7.4)	5 (4.6)	13 (12.0)	24(22.2)	34 (31.5)	14 (13.0)	4.8±1.8
Mean satisfaction with life score				24.4±7.3				

Discussion

Anxiety and depression have a major influence on life quality, adaption to treatment, perceived severity of the disease, and life satisfaction (4, 6, 42). This is the first study to examine cultural factors that may influence the anxiety, depression, and life satisfaction of lung cancer inpatients treated in a university hospital located in the Middle Black Sea region of Northern Turkey. This research was executed because health care professionals play an important role in the detection of anxiety and depression among patients diagnosed with lung cancer and in the provision of psychosocial support. In this study, it was detected that almost all the patients had a risk of anxiety and depression. They were partially satisfied with their lives.

In this study, the patients had a high risk of anxiety (97.2%) and depression (100%), and they were partially satisfied with their lives. Previous studies reported that the prevalence of depression varied between 9% and 87% in lung cancer patients (26-29, 31, 33, 35-37), anxiety prevalence varied between 10% and 43.3% (4, 26, 27, 29, 31, 33, 35). Studies also reported that when compared with other types of cancer types, the incidences of anxiety and depression prevalence were higher in those with lung cancer (28, 43, 44, 45). Research also showed that the anxiety and depression levels of patients differed before and after a diagnosis of cancer, with a decrease in anxiety levels and an increase in depression levels (33). According to one study, the prevalence of depression and anxiety was the same among inpatients and outpatients (29). Another study found that and depression and anxiety were much more common among patients with a diagnosis of nonsmall cell lung and depression was correlated with the patients' quality of life and health (26).

As physical functions decrease and symptoms increase, anxiety levels of patients also increase (4). Studies reported that anxiety affected the prognosis of the disease (26) and that the survival of patients who had depression was shorter than those who did not (35). Social support factors were obviously correlated with depression (37). The difference in the prevalence of anxiety and depression among lung cancer patients may be explained by the clinical and socio-demographic specifications of the patients, social support, and disease coping strategies.

In this study, the HADS-D subscale score of patients aged 64-76 was higher. The score was also higher in those whose incomes were higher than their expenditures, as well as in those who had social security and who were married and males. The findings of some studies are in agreement with the results of the present study. According to one study, the prevalence of anxiety and depression was higher among males than females with various cancers (prostate, urology,

colon, gastrointestinal, lung, head/throat, and brain) (46). Other studies found that anxiety and depression levels were significantly increased in patients younger than 65 years compared to those older than 65 years (31) and that individual characteristics, such as the patient's education level (26), working status (36), lifestyle behaviors (e.g., alcohol use and smoking) (26, 36) comorbidity (26), histological status and the clinical phase of the disease (26,31), did not affect anxiety and depression levels.

However, the findings of some other studies do not agree with those of the present study. Some found that depression and anxiety were higher in females (26, 44, 47), civil servants had the lowest depression score (19), and the self-employed ones had the highest depression score (19). Age group (26, 27, 36), sex (36), and marital status (26, 36) were reported not to affect anxiety and depression. Vodermaier et al. (2011) reported that anxiety and depression levels were higher in lung cancer patients than in patients with other cancers(47). They also reported that elders with cancer were less anxious and depressive than younger patients (47). Furthermore, they found that the stage of the disease was important in terms of the detection of depression in males but note females and that male patients with early phase lung cancer were less anxious and depressive than female patients and patients with advanced stage cancer. In the present study, the high level of anxiety and depression in the male patients may be explained by the high numbers of males in the sample. Furthermore, in the present study, the low anxiety and depression levels of the young patients compared to the patients over 65 years may be explained by the young patients believing they would recover from the disease and by them having a stronger commitment to life.

The majority of the patients (90.7%) were satisfied with their treatment, and they (74.1%) obtained a benefit from complementary and alternative treatments (74.1%), which they used to alleviate their symptoms. Most of the patients used biologically based therapies (herbal treatments, 69.4%; dietary changes, 44.4%) and mind-body control (prayer, 79.6%). Their use of complementary and alternative medicines points to dissatisfaction with their medical treatment and the desire to identify other treatment methods that would help them to manage their symptoms. A similar study found that lung cancer patients resorted to dietary/lifestyle changes (changing food options, changing former habits, and resting) to manage eating difficulties and oropharynx-related symptoms (48). The same study found that mind-body control (praying, listen to Buddha preaching) was the most popular complementary treatment used to manage fatigue, hair loss, numbness in fingers and toes, dyspnea, and taste changes. Another study of the symptoms, self-care, and life quality of Chinese-American cancer patients found that 20% of the patients used some type of Chinese herbal medicine (49). In a study by Gülgün and Kaya (2015) of a Turkish population, 56.5% of cancer patients reported obtaining a benefit from complementary and alternative treatments (mostly praying, 95%)(50). In that study, the patients stated that they used complementary and alternative treatments to relax and because they believed that they would enhance the effect of the chemotherapy treatment. In another study by Erbaycu et al. (2010), 27.4% of patients who used alternative medicines regularly while they were receiving chemotherapy reported obtaining a benefit. Of those patients, 8.0% consumed honey, 7.5% consumed nettle, 6.0% consumed carob/molasses, and 2.0% consumed plant juice tea, milk, or bee pollen(51). In a study by Düzen and Korkmaz (2015), 14.3%, of cancer patients used complementary and alternative treatment methods. The reasons they gave for their use of these methods included to combat the disease, inspire hope and positive thinking, reduce disease-relates symptoms, and increase the body's resistance to cancer. The same study found that although most of the patients used complementary and alternative treatment methods, they did not share this information with their doctors and nurses (52).

Today, complementary treatments play an important role in the control of cancer-related symptoms and treatment-related side effects. The literature also notes that cancer patients should be routinely questioned about their use of complementary treatments, as these can affect their medical treatment. However, one study emphasized that dietary supplements, which can potentially react with chemotherapeutic agents and herbal products, should not be used simultaneously with chemotherapy and radiation or before surgery (53). Although acupuncture can help in pain control

and decreasing painkiller drug level, information on the advantages and disadvantages of the alternative treatments should be given to the patient (53). Since complementary and alternative methods could have a positive/negative effect on the treatment applied to oncology patients, it is suggested that healthcare professionals need to be aware of the patient's cultural background, the way s/he interprets health/disease, the measures they use to get rid of disease, and avenues pursued for treatment and support (54).

In this study, many of the patients obtained a benefit from mind-body control, including praying. Religious practices, including praying, are an important way that the people apply for coping with the stressful situations in Turkey. As reported elsewhere, 99% of the Turkish population is made up of Muslims, and patients widely get solace from their religious beliefs, which help them to cope with the disease-related symptoms (50). Against the problems, seeking refugee of God allow for both making sense of the event and individuals' realizing opinions and feelings of the individuals due to the event. The individual who sees the problem he/she experiences will exceed his/her own self-sources seeks help. The tendency to seek refuge in religion and the support of God, showing patience and strength make contribution to that the individual feels him/her strong and copes with the problems. According to the literature, these kinds of religious-based coping practices increase the coping tenacity of people who may be physically and mentally unwell, and they provide inner peace, giving the individual the strength accept the disease and coping with the disease (23).

In this research, patients were partially satisfied with their lives, and life satisfaction was higher in patients aged 64-76 years old and in those whose incomes were higher than their expenditures. It was also higher among those who had social security and who were married and males. According to Sherlaw-Johnson et al. (2008), compared to the other cancer groups, life satisfaction was lower among lung cancer patients (42), as found in the present study. This finding may be related to the predicted short survival time of lung cancer patients compared to those with breast, prostate, and colorectal cancer and to treatment differences between the diseases (e.g., surgery, chemotherapy, and radiotherapy). In another study done by Bjordal et al. (1995) reported that 64% of cancer patients were satisfied with their lives, 9% were not satisfied, and the life satisfaction of the patients with cancer was lower compared to a control group (55). The same study found that stage of the disease and education level of the patient were correlated with the life satisfaction of cancer patients. These findings are in contrast to those found in the present study, which found that satisfaction of the patients who had a high education level was higher than that of the patients who had a medium and low education level.

In a study by Arslan et al. (2008), the life satisfaction of cancer patients was at a medium level, and it was higher than average in a literate group compared to secondary/high school graduated group (19). It was also higher in females than in males and higher among those who lived the provinces. Additional factors associated with higher than average life satisfaction were work status (i.e., a civil servant) and being from a nuclear family. In a study by Tate and Forchheimer (2002), life satisfaction was higher among elderly patients and married patients (56). Baker et al. (2007) reported that the life satisfaction of patients depended on relations with significant others (i.e.. friends, partners, children). The same study found that medical treatments, and various factors, such as the time since diagnosis, number of diagnoses, spread of the disease, treatment status, and cancer type, all affected their life satisfaction (57). The findings on the life satisfaction of the patients in the present study differ from those in the literature. The difference may be explained by the socio-demographic and clinical characteristics of the study group. A multidisciplinary approach, with oncology-psychiatry and social services, is needed to enhance the quality of life of lung cancer patients.

A cancer diagnosis is a stressful event, which often leads to fear and uncertainty. The anxiety and depression that commonly following a diagnosis of cancer can have a negative impact on life satisfaction. Disease- and treatment-related symptoms can damage the quality of life and functioning of the patient. These symptoms may have negative effects and interrupt the treatment. Understanding the risk factors for anxiety and depression and the factors that affect the life satisfaction of patients with lung cancer may aid the development of useful programs. The optimum management of the

disease and treatment related symptoms in the clinical environment depends on communication between the health care professionals and patients, a prompt diagnosis, and correct treatment of the symptoms (10).

Limitations

In this study, the data were collected using a survey form. A limitation of this study is that the findings were not based on simultaneously interviews with the patients. Future studies should include interviews with the patients. A long-term follow up to determine potential changes that may take place in the levels of anxiety, depression, and life satisfaction of the patients following their discharge from the hospital.

Conclusion

In this study, tiredness, taste changes, anorexia, vomiting, nausea, dyspnea, alopecia, pain, and weight loss were the symptoms most widely reported by the patients, and these depended on the cancer treatment and the patient's prognosis. The majority of the patients (90.7%) were satisfied with the treatment they received, and many patients (74.1%) obtained a benefit from complementary and alternative treatments, which they used to alleviate their symptoms. Many patients used biologically based therapies (69.4% herbal treatments, 44.4% dietary changes) and mind-body control (prayer, 79.6%). The socio-demographics of the lung cancer patients affected their levels of anxiety, depression, and life satisfaction. Based on the findings of this study, health care professional caring for lung cancer patients need to consider the treatment- and disease-related symptoms and side effects. They also need to provide psychological support, in the form of tailored programs, to these patients.

Declaration of authorship

All authors were responsible for the study conception/design, data collection/analysis, drafting of manuscript, critical revisions for important intellectual content, supervision, statistical expertise and administrative/technical/material support.

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Accepted: 8.3.2018
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EUR. J. ONCOL.; Vol. 22, n. 3-4, pp. 150-156, 2017

Geriatric evaluation in lung cancer care: a survey of daily practice

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Summary. *Background:* To identify ways to improve care for older lung cancer patients, we set out to examine how older lung cancer patients in the Netherlands are currently being analysed prior to oncological treatment and to explore the potential obstacles in the incorporation of a routinely performed geriatric evaluation. *Methods:* We sent a web-based survey to 138 Dutch pulmonologists specialized in lung cancer care between April and September 2015. *Results:* The response rate was 37%. According to the answers of the responding pulmonologist, a geriatric evaluation was available in 90% of the hospitals. This was performed routinely in a minority of the hospitals (45%) on the basis of age (18%), with use of some form of screening tool (27%), however mostly performed on ad hoc basis (56%). More than half (52%) of the respondents answered to be not, or not completely, satisfied with current geriatric evaluation. The main obstacles for implementing geriatric evaluation in standard care were lack of a structured format for this evaluation and lack of geriatric oncologic expertise. *Conclusion:* There is interest in the incorporation of a geriatric evaluation in the care for the heterogeneous elderly population with lung cancer. However, at the moment the optimal set-up for geriatric oncologic care is lacking. There seems to be no consensus about the optimal design in terms of patient selection, timing and use of screening tools. A closer collaboration between pulmonologists specialized in lung cancer care and geriatricians could help to improve appropriate care for elderly patients with lung cancer.

Key words: elderly, frail, geriatric assessment, lung cancer

Introduction

In the Netherlands, over 12,000 patients are diagnosed with lung cancer annually (1). Like elsewhere, half of these patients are over 70 years old, making lung cancer predominantly a disease of the elderly (1). The numbers of elderly patients are expected to rise in the next years due to prolonged life expectancy (2).

Many questions still remain unanswered regarding optimal lung cancer treatment for older patients. As ageing is an individual process that varies in comorbidity, remaining functional capacity, disabilities and geriatric conditions, treatment regimens investigated in fit, younger patients cannot automatically be extrapolated to older patients (3). Tailoring of care is mandatory, based on a thorough evaluation of the patient's overall health status in addition to tumour characteristics and preference of the patient. However, most physicians have never received specific training on the particular needs of older patients with cancer (4). Lack of this specific training can make them uncomfortable in decision-making for this population (4). In addition, elderly cancer patients have reported that their individual situation, including concurrent diseases and psychosocial status should receive more attention in the decision-making process (5).

Over the past years, international research groups have addressed this issue by advocating the incorporation of a geriatric evaluation into the standard oncological work-up to improve cancer care for older patients (3,6). A geriatric evaluation is used to assess the patient's health status across multiple domains (7). It can be used to identify previously unrecognized health issues which may guide treatment decisions and which can possibly be modified to improve quality of life and outcomes (8-10).

However, a geriatric evaluation in lung cancer practice is not yet implemented in standard care. It is unclear whether this is due to logistical issues such as insufficient time or personnel for performing the evaluation or insufficient support or priority among the involved professionals. Identifying these underlying obstacles could provide more clarity on the next steps that can be taken to improve lung cancer care for older patients.

The goal of our study was to examine how older patients with lung cancer are currently being evaluated prior to initiation of oncological treatment in the Netherlands and to explore the potential obstacles in the incorporation of a routinely performed geriatric evaluation.

Materials and methods

We developed an anonymous web-based survey and used software developed by SurveyMethods, Inc. (http://www.surveymethods.com). This questionnaire focused on the main issues related to geriatric evaluation in lung cancer care. The content of this survey is shown in Figure 1. Briefly, the first part of the questionnaire focused on the current methods of evaluating older lung cancer patients prior to oncological treatment. The second part focused on satisfaction with current practices in this treatment, possibilities for improvement and potential barriers for the incorporation of a geriatric evaluation. Questions ranged from multiple choices to open answers.

Between April 2015 and September 2015, this survey was sent to all 138 members of the Dutch Taskforce for Pulmonary Malignancies of the Dutch Lung Society (NVALT). We have sent the survey to their private e-mail address, the survey was only available via the link in the e-mail. The NVALT is the professional association for pulmonologists in the Netherlands. This taskforce consists of all NVALT members specialized in pulmonary malignancies.

No statistical analyses were performed only descriptive data are presented.

Results

Response rate and respondent characteristics

The overall response rate to the questionnaire was 37% (51/138). Characteristics of the respondents are listed in Table 1. Responses came from all over the country, covering 12 provinces of the Netherlands and a range of hospital types, including primary, secondary and tertiary referral centres were represented.

Geriatric evaluation in daily lung cancer practice

According to the answers of the respondents to this survey, in 90% of the hospitals some form of geriatric evaluation is performed, ranging from an occasional, ad hoc assessment to a routine assessment of all oncologic patients aged 70 years or older. As visualized in Figure 2, the way that patients are selected for a geriatric assessment differs. In 56% the pulmonologists or oncologic specialized nurses refer patients as needed based on their own clinical judgement or based on the opinion of the multidisciplinary team for lung cancer treatment. On the other hand, 18% of the respondents answered that patients are routinely referred when reaching a particular age. Other methods for patient selection include some form of frailty screening tool (15%), the Geriatric Navigator (6%)(11) – a Dutch web-based instrument for assessing overall health status and the presence of particular geriatric impairments, developed specifically for older cancer patients – and 6% used a combination of these tools. In addition, in some hospitals non-specialized nurses or were involved in this selection.

As the way that patients are selected for a geriatric evaluation differs, the involved healthcare professionals for the geriatric evaluation selection process differ

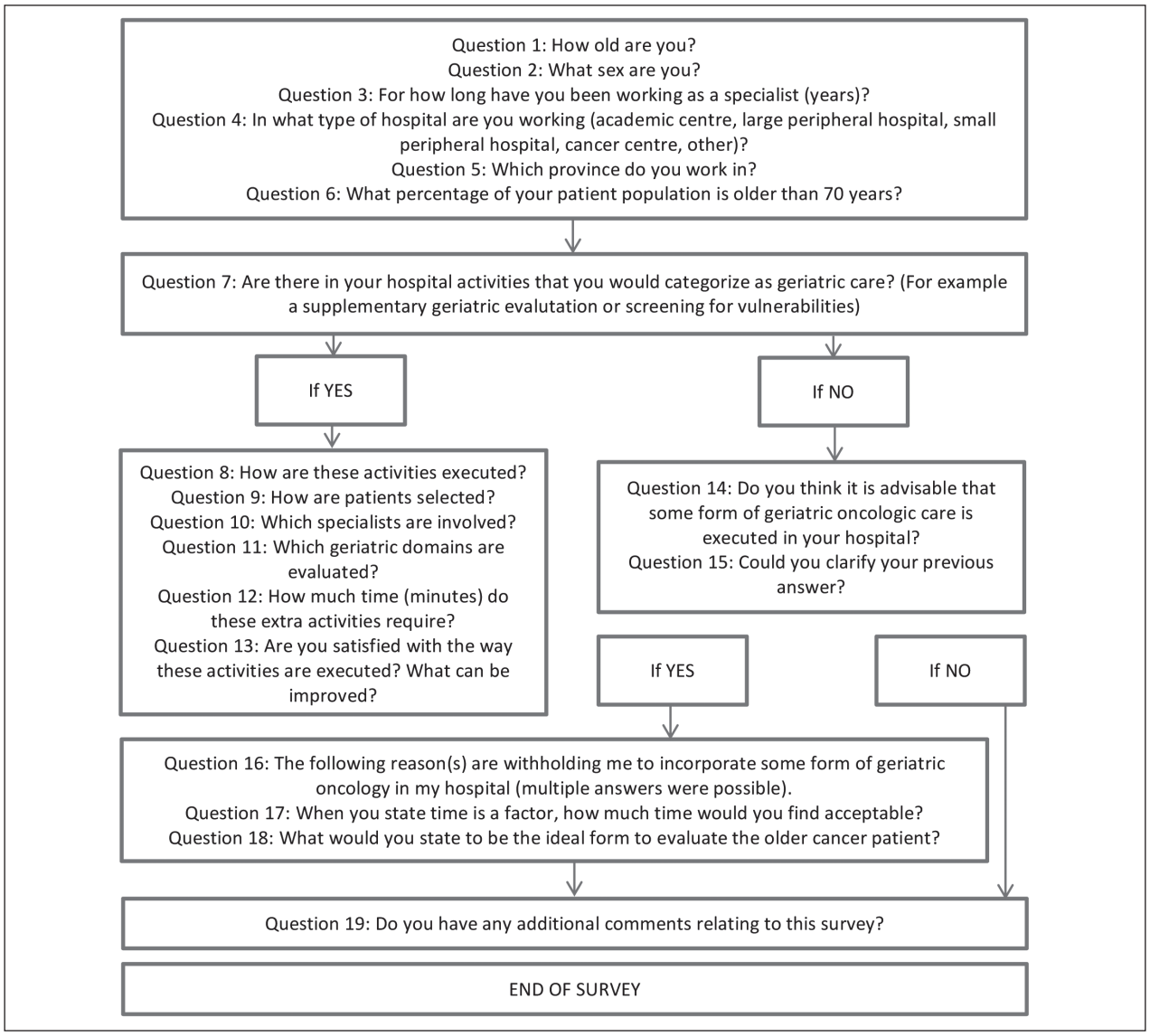

Figure 1. Content of survey (translated from Dutch)

as well. There is a wide range of professionals involved in this process ranging from geriatricians (74%), oncologic specialized nurses (68%), geriatric specialized nurses (32%), physiotherapists (6%) to psychiatrists and psychiatric nurses (9%).

When geriatric evaluations are being performed – routinely or ad hoc – 45% of the respondents reported that at least four different geriatric domains are examined and 35% examine eight domains or more. Domains that are most frequently investigated, besides comorbidity and polypharmacy, are nutritional status (81%), activities of daily living (71%), cognition (68%) and social network (68%). Instrumental activities of daily living (32%) and mood (48%) were the least examined domains. The median time that a geriatric evaluation requires is reported as 20 minutes, with a range between 1 and 120 minutes.

Satisfaction with current practices

The respondents who reported to have implemented a form of geriatric evaluation for their elderly

Table 1. Characteristics of respondents

	Total (n=51)
Response rate	51/138 (37%)
Median age of respondent (range)	49 (33-61)
Years of experience as medical specialist (range)	11 (0-28)
%female	30%
Type of hospital	
Academic	12%
Large peripheral	64%
Small peripheral	22%
Tertiary/categorical	2%
Median % patients over 70 years old	50% (20-80)

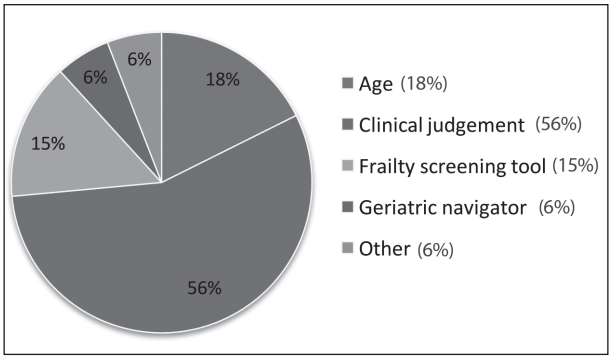

Figure 2. Selection of patients for geriatric assessment

cancer patients were asked how satisfied they are with current practice. One-quarter stated to be completely satisfied. Over half (52%) answered that they are not, or not completely, satisfied with the way the geriatric evaluation is performed in their hospital at the moment .The primary issue – as reported by 2/3 of these respondents – is the lack of a consistent, structured set-up for the geriatric evaluation. Many pulmonologists declared that they struggled with finding the right format and a lack of experience with available screening tools. There seems to be no consensus about the design of this evaluation, about the patient selection, the timing, the focus of geriatric domains, the use of screening tools and the required action that need to be taken following the geriatric evaluation.

Another issue that was mentioned was the oncologic expertise of the geriatricians in their hospital: 19% of the dissatisfied respondents answered that the geriatricians only provide general recommendations but are lacking specific expertise in the treatment or decision-making for older cancer patients.

A third issue is the extra costs of this evaluation, as described by 10% of the dissatisfied respondents. They answered that they are worried about the efficacy and economic issues of health care.

Discussion

Lung cancer is often diagnosed in advanced stages, generally progresses rapidly, and is mainly a disease of elderly patients (1). As the elderly represent a heterogeneous population, special attention and tailoring of care is needed for this patient population (12). This study provides an insight in the current use of geriatric evaluation of lung cancer patients in the Netherlands and describes the encountered obstacles for implementation of standard geriatric oncologic care in patients with pulmonary malignancies. According to the answers of the responding pulmonologist, a geriatric evaluation is available in 90% of the hospitals. This is performed routinely in a minority of the hospitals on the basis of age (18%) or with use of some form of screening tool (27%) and mostly performed on ad hoc basis (56%). More than half (52%) of the respondents answered to be not, or not completely, satisfied with current geriatric evaluation of their patients. The main issue is the lack of a structured format, which is considered mandatory for incorporation of a geriatric evaluation in oncologic care and the decision making process.

A recent survey about geriatric oncologic care among Dutch cancer specialist (surgeons, radiotherapist, medical oncologist and geriatricians) showed comparable outcomes as described in our study (13, 14).They declared that the use of geriatric evaluations in elderly cancer care was confirmed by half of the respondents, varying from 65% of medical oncologist tot 27% of radiation oncologists (13). It was routinely performed in one third of the patients; in another third the geriatric evaluation was performed on an ad hoc basis only and the remaining third did not elaborate on its execution. Cancer specialists seem to be inter-

ested in introducing a geriatric oncology program and a closer collaboration with geriatricians (15). However, a lack of priority and uncertainty of the optimal set-up for a geriatric oncology program remain important obstacles (13-15).

At the moment, treatment decisions in lung cancer care are based on clinical assessment in combination with age and performance status discussed at the multidisciplinary tumour board meeting. However, as ageing is an individual process, chronological age does not necessarily reflect one's biological age (12). In addition, age is not found to be predictive for survival of elderly lung cancer patients (16, 17). While performance status has a significant association with survival, it has been suggested that within the elderly population, performance status alone is insufficient in discriminating between fit and vulnerable patients (3).

The identification of frail patients can be improved by using a geriatric assessment. However, the relevance of a geriatric assessment in lung cancer care has not been extensively researched. Geriatric impairments are highly prevalent, even in patients with good performance status, and are of prognostic significance (17-23). In particular, impairments in objectively measured physical capacity and impairments in nutritional status are predictive of early mortality (16-18, 21, 22, 24). Furthermore, the information revealed by a geriatric assessment can lead to changes in oncologic treatment choices as well as non-oncologic interventions (25, 26). In addition, a geriatric assessment-stratified treatment allocation can potentially decrease overall toxicity and aggressiveness of treatment without decreasing efficacy (27). Thus, there are sustainable arguments for the implementation of geriatric assessments in pulmonary oncology.

At the moment little is known about the effects of applying guideline recommended treatment in elderly cancer patients. An analysis of the NIH trial registry showed that elderly patients and those with comorbidities are often excluded from participation in clinical trials (28). We do take a risk when we apply these treatments on frail and elderly patients. More research that includes these patients is urgently needed.

This study has several limitations. First, we used open-ended questions to give the respondents the opportunity to freely provide their input. However, this required a secondary interpretation and categorization of answers. We tried to make this interpretation as objective as possible by using a mix between open-ended and pre-formulated answers. Second, the response rate was only 37%, which is a well-known issue in survey-based studies. In addition, it is not unlikely that those pulmonologists with special interest for geriatric oncology answered this survey, which makes it unclear if these answers are representative for all oncologic pulmonologists. Despite these limitations, this is the first study that provides information about the use and the encountered obstacles for a geriatric evaluation in lung cancer patients.

A suggestion to improve geriatric evaluation in lung cancer patients would be an intensified cooperation between lung cancer specialists and geriatricians, for example by including a geriatrician in the multidisciplinary tumour board meetings. At these meetings patient centred information is often lacking and the available information is mainly disease specific (29). Knowledge on physiological ageing, remaining functional capacity in combination with comorbidity is of major importance for the assessment of a patient's ability to tolerate treatment (29). The presence of geriatricians at the MDT can lead to increased patient-centred decision-making (30). However, in addition to the urge of specific training of oncologists on the particular needs of elderly cancer patients, geriatricians need a specialized training in oncological care as well (4). Only a quarter of the responding geriatricians in the survey among Dutch cancer specialists reported that elderly cancer patients received a routinely performed geriatric evaluation prior to the initiation of oncologic treatment, and unfortunately many geriatricians reported that optimising cancer care for elderly patients was currently not a priority at their centre (14). Given the significant burden and complexity of cancer for the elderly, geriatricians are encouraged to share their expertise with other specialists to be able to optimise care for elderly cancer patients (14). The cooperation between pulmonologists and geriatricians only has an additional value if they both exactly know what their role is and if there is a format of what may be expected from their consultation (15).

Conclusion

There is interest among oncologic pulmonologists in the incorporation of a geriatric evaluation in the care for the heterogeneous elderly population with lung cancer. However, at the moment a structured format of a geriatric evaluation for this category of patients is lacking. There is no consensus about the optimal design of this evaluation in terms of patient selection, timing, use of screening instruments and the required action that need to be taken following the geriatric evaluation. A closer collaboration between lung cancer specialists and geriatricians could help in bridging the gap between geriatrics and oncologic care to optimize the treatment of lung cancer in elderly patients.

Financial support: this study was supported by the Aart Huisman Scholarschip for research in geriatric oncology and the Cornelis Visser Foundation.

Author's contributions:
KJGS: made substantial contribution to conception and design, acquisition, analysis and interpretation of data, drafting the article and final approval of the version to be published.
MEH: made substantial contribution to conception and design, analysis and interpretation of data, revising the article critically for important intellectual content and final approval of the version to be published.
JWJL: made substantial contribution to conception and design, revising the article critically for important intellectual content and final approval of the version to be published.
LJRvE: made substantial contribution to conception and design, revising the article critically for important intellectual content and final approval of the version to be published.

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Received: 20.07.2016
Accepted: 14.11.2017
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EUR. J. ONCOL.; Vol. 22, n. 3-4, pp. 157-158, 2017

HPV and cancer. Is this all Eve's fault?

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Dear Editor,

"Eve told Adam she had eaten the fruit. She would have to leave the garden. She gave Adam some of the fruit. Adam ate it" (Genesis 3:6-7). As symbolized in Genesis (and a long time before in the Hesiod's telling of Pandora's tale (Hesiod, Erga kai hemera, …) Eve is renowned for being guilty for the original sin and the cause of the fall from the Garden of Eden. Human papillomavirus (HPV) infection and transmission rates differ by gender, with the rates of sexual transmission from women to men substantially higher than from men to women (1). Due to negativity surrounding Eve, women have been subject to intense scrutiny through history. Women's life experiences, such as reproductive processes and sexuality, have been undeniably more medicalized than men's through the ages (2). The modern history of HPV is an excellent launching point for the discussion around the sociological implications of gender medicine. Women's body scrutiny is already well attested in ancient Greek medical and philosophical thought (i.e. in the Corpus Hippocraticum as well as in the later Aristotelic thought); the conceptualization of female bodies as defective and 'guilty' ones was maintained through ages in Western culture as a means of social control, through the ideological binding around venereal diseases and women actions. Diseases associated with sexuality have been historically categorized as feminine. The development of HPV vaccines, which prevent a range of HPV-related cancers, is a remarkable story of scientific achievement. The nonavalent HPV vaccine has been recently approved, providing increased protection in addition to the quadrivalent HPV vaccines, already commercially available in a large number of countries (3). HPV is not gender- specific. Nevertheless, the renowned causal association between HPV and cervical cancer and the historical medicalization of women's sexuality inevitably led to the feminization of HPV, with profound implications on community awareness and primary prevention strategies. The HPV vaccine was licensed for use by the US FDA in female adolescents in 2006, while a routine recommendation for males was only made in 2011 (4). The HPV vaccine history timeline illustrates the false theory of the HPV herd immunity, which hypothesized that females HPV adequate vaccination would also protect males. Intrinsic limits of this theory lie in the unexpected low rate of vaccination among young females and in the unrecognized proportion of men having sex with men. The feminization of HPV, which ultimately discriminates against females and males through different processes, has counterintuitive negative consequences on men's health. HPV causes cancer in both men and women. The HPV-related cancer burden remains higher in women than men, primarily owing to cervical cancer. Nevertheless, epidemiological gender disparities in HPV- related cancers may be significantly reduced over time in countries that have implemented effective cervical cancer screening programs. Additionally, anal cancer incidence is increasing in recent years, with higher rates in men having sex with men, and a dramatic rising trend is being observed among men in HPV-related oropharyngeal cancer incidence By contrast, HPV testing for screening and the understanding of precancerous lesions in anatomic sites other than the

cervix are still in their relative infancy (5). Although women continue to suffer inequalities in education, labor market or political representation, gender-based disparity in health mainly disadvantage men. Major global health institutions, including WHO, report substantially worse health outcomes among men and a significant gap in life expectancy between men and women (6). Research has begun to investigate how the sociological construct of gender may influence health, identifying a causative association between masculinity and health disparity between men and women. Literature reveals the interaction between gender and health promotion, being women more likely than men to get preventive screening and to use health services. The cultural imprint of women's body scrutiny, in the HPV framework, paradoxically results in women health promotion and discrimination against men. In the attempt to move from a mere description to medical intervention, men-centered awareness campaign and screening programs should be encouraged. Women have traditionally been underrepresented in clinical trials and early-stage medical studies tend to use male animals more than female ones. Research has demonstrated that gender bias has significant implications for the quality of science itself (7). The NIH (National Institutes of Health) has recently launched a plan to address the issue of gender inequalities across biomedical research and released policies that require the balance of male and female cells and animals in preclinical studies. Throughout history, research has been essential to major global health achievements and, especially in the last century, research contributed in promoting health through the elimination or control of vaccine-preventable diseases. The feminization of HPV and the gender bias in HPV screening and vaccination might have hampered the knowledge and hindered the progress against HPV-related cancers overall. The recognition of HPV gender neutrality is essential to get out of the old and questionable labeling of women as carrier of sexually transmitted diseases and to promote gender equity in health care.

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Received: 15.2.2018
Accepted: 20.2.2018
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