Expressional Analyses of *NM23-H1, KAI1* and *MKK4* Metastasis-Related Genes in Metastatic Ovarian Carcinomas

Metastatik Over Karsinomlarında Metastazla İlişkili *NM23-H1, KAI1* ve *MKK4* Genlerinin Ekspresyon Analizleri

Türker BİLGEN, PhD,[a]
Gülgün ERDOĞAN, MD, Assoc.Prof.,[b]
Tayup ŞİMŞEK, MD, Prof.,[c]
Hakan GÜLKESEN, MD, Assis.Prof.,[d]
Elif PESTERELİ, MD, Prof.,[b]
Şeyda KARAVELİ, MD, Prof.,[b]
Güven LÜLECİ, PhD, Prof.,[a]
İbrahim KESER, PhD, Prof.[a]

Departments of
[a]Medical Biology,
[b]Pathology,
[c]Gynecology and Obstetrics,
[d]Biostatistics and Medical Informatics,
[e]Medical Informatics,
Akdeniz University Faculty of Medicine, Antalya

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Yazışma Adresi/*Correspondence:*
İbrahim KESER, PhD, Prof.
Akdeniz University Faculty of Medicine, Department of Medical Biology, Antalya,
TÜRKİYE/TURKEY
keser@akdeniz.edu.tr

ABSTRACT Objective: Cytoreductive surgery as a basis of therapy in epithelial ovarian carcinomas (EOC) provides the primary tumor and metastatic tumor samples from a same patient. This gives an excellent opportunity for evaluation of metastatic factors by excluding inter-individual differences. Therefore, we aimed to define changes at mRNA levels of *NM23-H1, KAI1* and *MKK4* metastasis-related genes in the paired normal tissue, primary tumor and omental metastatic tumor samples obtained from a same patient. **Material and Methods:** mRNA levels were quantified by quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) following total RNA extraction in normal tissues, primary malign tissues of EOC, and its metastatic lesions on omentum for 41 patients with stage III-C (FIGO) EOC. **Results:** We found that mRNA level of *NM23-H1* was significantly higher in metastatic samples compared to primary tumors (p=0.009). On the other hand, *MKK4* was found to be significantly lower in primary tumor samples compared to normal tissues (p=0.024). There was no significant change at mRNA level of *KAI1* among normal tissues, primary tumors and omental metastatic tumor samples. **Conclusion:** We suppose that in detailed functional studies, approaches that suppress *NM23-H1* gene and restore *MKK4* gene would make these genes important molecular targets for treatment of metastatic EOC in the future.

Key Words: Genital neoplasms, female; neoplasm metastasis; *NM23-H1; KAI1; MKK4;* omentum

ÖZET Amaç: Epitelyal over karsinomunda (EOK) tedavinin temelini oluşturan sitoredüktif cerrahi, aynı hastadan hem primer tümör hem de metastatik tümör dokularının elde edilmesine olanak sağlar. Böylelikle bireyler arasındaki farklılıkların dışlanmasıyla metastatik faktörlerin değerlendirilmesi açısından mükemmel bir fırsat sağlanmış olur. Bu nedenle bu çalışmamızda; aynı hastadan elde edilen primer tümör, metastatik tümör ve normal dokularda metastaz ile ilişkili *NM23-H1, KAI1* ve *MKK4* genlerinin mRNA düzeylerindeki değişimleri belirlemeyi amaçladık. **Gereç ve Yöntemler:** Evre III-C (FIGO) EOK tanısı almış 41 hastada, mRNA düzeyleri aynı hastadan cerrahi yöntem ile elde edilen primer tümör, ondan köken alarak omentum üzerine implante olan metastatik lezyonlar ve normal dokulardan total RNA izolasyonunu takiben kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (Q-RT-PCR) yöntemi kullanılarak belirlenmiştir. **Bulgular:** *NM23-H1* mRNA düzeyinin metastatik tümör dokularında primer tümör dokularına kıyasla anlamlı derecede daha yüksek olduğunu bulduk (p=0,009). Diğer yandan, *MKK4* mRNA düzeyinin primer tümör dokularında normal dokulara kıyasla anlamlı derecede daha düşük olduğu belirlendi (p=0,024). Ayrıca *KAI1* geninin mRNA düzeyinde normal dokular, primer tümör ve metastatik lezyonlar arasında anlamlı bir değişim olmadığı belirlendi. **Sonuç:** Gelecekte yapılacak detaylı fonksiyonel çalışmalarla, *NM23-H1* geninin baskılanması ve *MKK4* geninin yeniden ekspresyonunun sağlanması yaklaşımları ile bu genlerin metastatik EOK'nın tedavisi için önemli birer moleküler hedef olabileceklerini düşünmekteyiz.

Anahtar Kelimeler: Genital tümörler, kadın; tümör metastazı; *NM23-H1; KAI;, MKK4;* omentum

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Epithelial ovarian carcinoma (EOC) is the leading cause of death among gynecological malignancies. EOC is generally diagnosed at advanced stages in which tumor has already spread into abdomino-

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pelvic cavity.[1] At the time of diagnosis, 70-80% of patients with EOC have metastatic disease which is one of the major reasons of high mortality rate together with chemoresistance in EOC.[2,3] Metastasis is a complex and multi-step process which has not been completely elucidated yet. Currently, there are more than 20 experimentally described metastasis suppressor genes which have been defined according to their ability to inhibit metastasis in secondary sites without affecting tumor growth at the primary site.[4]

NM23-H1, as a nucleoside diphosphate (NDP) kinase, was the first identified metastasis suppressor gene discovered in 1988. In addition to NDP kinase activity, 3'-5' exonuclease activity has been reported for *NM23-H1*.[5] Metastasis suppressor role of *NM23-H1* has been shown in various cancer types with diverse series of approaches testing metastatic potential.[6] While reduced NM23-H1 protein level has been associated with peritoneal implants, over-expression has been related with distant metastasis of ovarian serous carcinoma.[7] Despite *NM23-H1* has been considered as a metastasis suppressor, it has been described as a good prognostic marker in ovarian cancer and also as a poor prognostic marker in some other studies.[7,8] Because of conflicting results mentioned above, the role of *NM23-H1* in ovarian carcinoma is still unclear.[9]

The *KAI1/CD82* gene, located at human chromosome 11p11.2 region, encodes a plasma membrane glycoprotein of 267 amino acids and belongs to the transmembrane 4 superfamily (TM4SF, Tetraspanins).[10] Anti-metastatic potential of *KAI1/CD82* has been suggested for a wide range of malignancies including ovarian cancers.[11,12] Mitogen-activated Protein Kinase Kinase 4 gene (*MKK4 / SEK1* / JNKK1 also known as Stress-activated Protein or Erk Kinase 1) as a dual Ser/Thr kinase, is located at chromosome 17 in human genome and encodes a protein of 399 amino acids, directly phosphorylates and activates the c-Jun N-terminal kinase (JNK) and p38 in response to diverse set of environmental stresses and extracellular stimuli.[13,14] In addition to prostate cancer where *MKK4* has been first described as a metastasis suppressor, declining protein level of *MKK4* from normal ovarian surface epithelium to tumor and metastatic samples has been shown in ovarian cancer.[15]

In this study, we aimed to define changes at mRNA levels of *NM23-H1, KAI1*, and *MKK4* metastasis-related genes among paired normal tissues, primary tumors and omental metastatic tumor samples obtained from the same individuals including 41 stage-IIIC EOC patients in order to evaluate their roles in metastasis of EOC.

MATERIAL AND METHODS

PATIENTS AND TISSUE SAMPLES

This study includes 41 EOC patients with stage IIIC tumors according to International Federation of Gynecology and Obstetrics (FIGO) staging system, who underwent primary surgery because of epithelial ovarian carcinoma without receiving preoperative chemotherapy in the Medical Faculty Hospital of Akdeniz University. Medical Faculty of Akdeniz University Ethics committee has approved the study and all patients provided written informed consents. They all had histologically-confirmed malignant epithelial ovarian tumors first by pathologists with frozen examination during the operation and then routine histopathological examination of the dissected tumor samples. Tissue samples were obtained as fresh samples for 31 patients and as formalin fixed paraffin-embedded (FFPE) archival surgical specimens for 10 patients. For FFPE samples, paraffin blocks from the best slides of tumor tissue were selected by an expert pathologist. All studied tumor samples were subjected to histopathological examination to minimize non-tumor tissue by an expert gynecological pathologist. We used fallopian tube in our study as the normal control rather than normal ovarian surface epithelium because it is difficult to obtain normal ovarian surface epithelial cells. The other reason is also that EOC arises from epithelial cells and epithelial cell content is very high in the fallopian tube.

GENE EXPRESSIONS WITH QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (Q-RT-PCR)

Gene expression patterns of *NM23-H1, KAI1, MKK4* genes in surgically obtained normal tissues

(n=38), primary malignant epithelial ovarian tumors (n=41) located in the ovary, and its metastates in omentum (n=35) were analyzed in 41 patients with Q-RT-PCR using TaqMan probe system following cDNA synthesis by TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Total RNA extractions were performed by Trizol (Sigma, Germany) for fresh samples and by Recover All Total Nucleic Acid Isolation Kit (Ambion Inc., Austin, TX, USA) for FFPE tumor sections. Q-RT-PCR analyses were performed using the ABI 7500 Real Time PCR System (Perkin Elmer Applied Biosystems, Foster City, CA, USA). All samples were tested in duplicate wells. PCR was carried out with the TaqMan Universal Master Mix, primer-probe mix for each *MKK4* (Hs 00387426_m1, Applied Biosystems, Foster City, CA, USA), *NM23-H1* (Hs00264824_m1, Applied Biosystems, Foster City, CA,USA), and *KAI1* (Hs00174463_m1, Applied Biosystems) genes, 4 microlitres of cDNA in 20 microlitres final reaction volume. 18S ribosomal RNA (TaqMan Ribosomal RNA control reagents kit, Applied Biosystems, Foster City, CA, USA) primer-probe mix has been used as an internal control gene in each PCR well as multiplex PCR reaction.

STATISTICAL ANALYSIS

Wilcoxon signed ranks test was used to compare mRNA levels among normal tissue, primary tumor and metastatic tumor samples. Statistical analyses were performed using SPSS 15.0 software (SPSS, Inc., Chicago, Illinois) with $p < 0.05$ considered significant.

RESULTS

Clinico-pathological data of the patients are shown in Table 1. The cycle threshold (CT) values were normalized to normal tissues for primary tumor and normalized to primary tumors for metastatic samples using internal control gene (18S ribosomal RNA). All CT values indicating mRNA levels of *NM23-H1*, *KAI1* and *MKK4* genes in normal tissue, primary tumor and omental metastatic lesions are shown in Figures 1 and 2. When we compared the normalized CT values for *NM23-H1*, *KAI1* and *MKK4* genes among normal tissues, primary ovarian tumors and their omental implants, we found that *NM23-H1* mRNA level was significantly

TABLE 1: Clinicopathological data of patients with epithelial ovarian carcinoma.

Histologic subtypes	Number of patients
Serous	19
Endometrioid	10
Mixed type (endometrioid + serous)	6
Mixed type (clear cell + serous)	2
Mucinous	3
Borderline, Mucinous	1
Clinical Stages (FIGO); Stage IIIC only	
Age (Mean ± SD, years); 58.8 ± 10.5	
Lymph node status; Negative: 15	
Positive: 26	

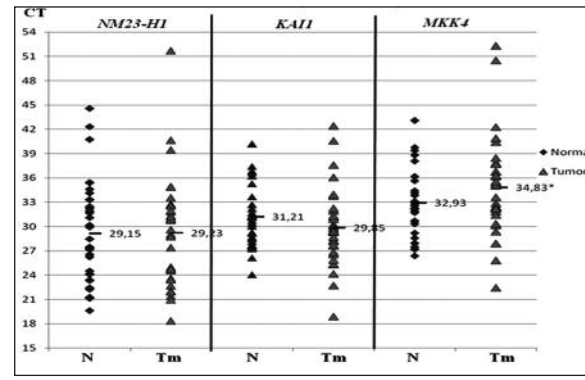

FIGURE 1: Comparison of the normalized cycle threshold values between normal tissue (N) and primary tumor samples (Tm).
*p<0.05, mRNA level of MKK4 in primary tumor samples is statistically different compared to normal tissue.

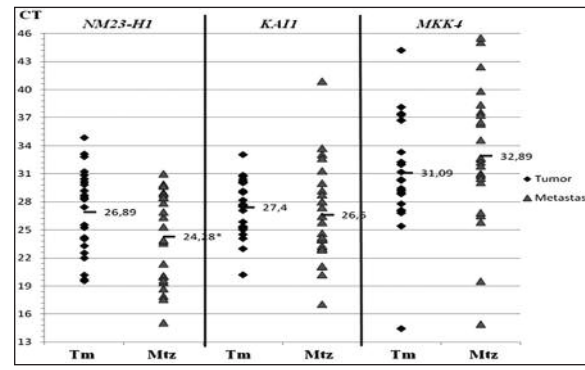

FIGURE 2: Comparison of the normalized cycle threshold values between primary tumor (Tm) and metastatic tumor samples (Mtz).
*p<0.05, mRNA level of NM23-H1 in metastatic samples is statistically different compared to primary tumor.

higher in the metastatic samples compared to primary tumors (p=0.009) (Table 2 and Figure 2) and *MKK4* mRNA level was significantly lower in primary tumor samples compared to normal tissues (p=0.024) (Table 2 and Figure 1). The median and min-max CT values and p values obtained by comparison of the CT values of these three genes in paired normal tissue-primary tumor and primary tumor-metastasis are shown in Table 2.

DISCUSSION

Ovarian cancers have the highest mortality rate among the gynecological cancers due to late diagnosis since the tumor has already metastasized in most of the cases.[16] In this study, therefore, we investigated the mRNA levels of *NM23-H1, KAI1* and *MKK4* metastasis-related genes as potential targets in omental metastasis of EOC in paired clinical samples by excluding inter-individual differences.

Along with determination of genomic alterations in metastatic lesions compared to primary tumor tissue, global gene expression profiling could help us to identify metastasis-related genes. However, differential expression of *NM23-H1, KAI1* and *MKK4* genes has not been reported in primary ovarian tumors compared to metastatic lesions of EOC in two recent global gene expression analyses.[17,18] On the other hand, metastasis suppressor roles of these three genes in ovarian cancer have been speculated by previous studies at the protein level.[7,12,15,19] A summary of the studies investigating the expression levels of *NM23-H1, KAI1* and *MKK4* genes in clinical samples are given in Table 3.

In our study, we found that the mRNA levels of *NM23-H1* and *KAI1* genes were increased in primary and metastatic tumor samples. However, only the change for *NM23-H1* in metastatic tumor samples was statistically significant when compared to primary tumor. Despite *NM23-H1* and *KAI1* genes have been described as metastasis suppressors, increased protein levels of these two genes in EOC have also been reported before.[7] On the other hand, due to lack of functional experimental data, there is no further explanation regarding their suppressor roles either in metastasis or in carcinogenesis of EOC. Nevertheless, our data showing increased mRNA levels of *NM23-H1* and *KAI1* genes in EOC may contribute to elucidate previous uncertain situations of these two genes in EOC.

Loss of functional mutations in *MKK4* has been reported in approximately 5% of a wide variety of human tumors and it has also been described as a tumor suppressor in the ovarian cancers.[20-24] Contrarily, it has been reported that *MKK4* acts as a pro-oncogene instead of being a suppressor in breast and pancreatic tumors.[25] It is not still clear whether *MKK4* suppresses or promotes carcinogenesis. Our results may imply that *MKK4* acts as a tumor suppressor in EOC since we showed decreased mRNA level of *MKK4* in tumor samples compared to normal tissues. It can be speculated that *MKK4* may have two opposite roles in differ-

TABLE 2: Comparison of the normalized cycle threshold values of primary tumors with paired normal tissues and metastatic tumor samples with paired primary tumors.

Metastasis related genes	Normal tissue median (min-max)	Primary tumor median (min-max)	n	P	Primary tumor median (min-max)	Metastasis median (min-max)	n	p
NM23-H1	28.0 (19.6-44.6)	29.3 (18.4-51.7)	34	0.720	27.9 (19.5-34.9)	24.6 (15.1-31.0)	24	0.009*
KAI1	30.7 (24.1-40.2)	29.4 (18.9-42.4)	33	0.104	27.5 (20.2-33.0)	25.8 (17.0-40.9)	25	0.162
MKK4	32.3 (26.4-43.1)	35.0 (22.4-52.3)	33	0.024*	30.3 (14.5-44.2)	32.4 (14.9-45.5)	24	0.110

* p<0.05 is considered significant. min-max: Minimum-maximum.

TABLE 3: Literature review of *NM23-H1, KAI1* and *MKK4* genes, and comparison with our results.

Metastatic genes	Literature	Youn et al.[27]	Tas et al.[8]	Arık et al.[28]	Present study
NM23-H1	Material	86 ovarian serous carcinomas	50 ovarian carcinomas	71 benign, borderline and malignant ovarian serous carcinomas	41 metastatic epithelial ovarian carcinomas
	Method	IHC	IHC	IHC	Q-RT-PCR
	Result	Variable protein level	Variable protein level	Increased protein level	Increased mRNA level in metastatic tumor samples
KAI1	**Literature**	**Liu et al.[29]**	**Houle et al.[30]**	**Schindl et al.[31]**	
	Material	102 benign, borderline, primary invasive, metastatic and recurrent epithelial ovarian carcinomas	32 primary and 8 metastatic ovarian epithelial carcinomas	107 epithelial ovarian carcinomas	41 metastatic epithelial ovarian carcinomas
	Method	IHC, RT-PCR	IHC	IHC	Q-RT-PCR
	Result	Lower protein and mRNA levels	High protein level in low-grade and low protein level in high-grade	Lower protein level	No significant change detected
MKK4	**Literature**	**Yeasmin et al.[23]**	**Yamada et al.[32]**	**Spillman et al.[33]**	
	Material	93 benign, borderline and ovarian carcinomas	34 metastatic ovarian carcinomas	24 ovarian carcinomas	41 metastatic epithelial ovarian carcinomas
	Method	IHC	IHC	IHC, WB	Q-RT-PCR
	Result	Lower protein level	Lower protein level	Lower protein level in surgically sub-optimally removed samples	Lower mRNA level in primary tumor samples

IHC: Immunohistochemistry, WB: Western blotting, Q-RT-PCR: Quantitative real time polymerase chain reaction.

ent cancer types. In addition to the role of *MKK4* in carcinogenesis, it has also been described as a metastasis suppressor in ovarian carcinoma by functional experimental studies.[15,19] Our results confirm previous studies by showing low *MKK4* mRNA level in omental metastatic implants compared to primary tumor tissues since that result may support its metastasis suppressor role in EOC.

Despite some remarkable results regarding the role of *NM23-H1* and *MKK4* genes in ovarian carcinomas were obtained, our study was only limited to expressional analyses of three metastasis-related genes at RNA level. Therefore, further functional and protein based studies are warranted for a more detailed evaluation of clinical relevance of the differences detected at RNA level in this study.

There are no enough data to classify the genes involved in metastatic process according to their roles and cancer types. Identification of candidate genes which are possibly related with metastatic event in a particular cancer type may reveal new specific targets for anti-cancer or rather anti-metastatic therapy.[26] Thereby, it is conceivable that targeted therapy aiming these genes in EOC could improve the patients' responses. In consequence, we conclude that *MKK4* may be involved in both tumorigenesis and metastasis in EOC making it quite important for new strategies targeting EOC. *NM23-H1* might be another target to be suppressed to inhibit the metastatic growth. Additionally, our findings showing up-regulation of *KAI1* and *NM23-H1* may contribute to clarify previous uncertain situations regarding the roles of these genes in metastasis of EOC.

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