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RESEARCH

HGF/c-Met Overexpressions, but not Met Mutation, Correlates with Progression of Non-small Cell Lung Cancer

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Abstract Hepatocyte Growth Factor (HGF) and its receptor c-Met are suggested to play an important role in progression of solid organ tumors by mediating cell motility, invasion and metastasis. Overexpression of HGF and c-Met have been shown in non-small-cell lung cancer (NSCLC). However, their role in tumor progression is not clearly defined. The aim of this study is to determine the role of HGF/c-Met pathway and its association with invasion related markers and clinicopathologic parameters in NSCLC. Immunohistochemical analysis was performed on 63 paraffin-embedded NSCLC tumor sections. The expressions of invasion related markers such as Matrix Metalloproteinases (MMPs) 2 and 9, Tissue Inhibitor Metalloproteinase (TIMP) 1 and 3 and RhoA were also examined. Co-expression of HGF/c-Met was significantly associated with lymph node invasion and TIMP-3 and RhoA overexpressions. There were positive correlation between TIMP-3 overexpression and advanced stage and negative correlation between RhoA overexpression and survival. DNA sequencing for Met mutations in both nonkinase and tyrosine kinase (TK) domain was established. A single nucleotide polymorphism (SNP) in sema domain and two SNPs in TK domain of c-Met were found. There was no statistically significant correlation between the presence of c-Met alterations and clinicopathologic parameters except shorter survival time in cases with two SNPs in TK domain. These results suggest that HGF/c-Met might exert their effects in tumor progression in association with RhoA and probably with TIMP-3. The blockade of the HGF/c-Met pathway with RhoA and/or TIMP-3 inhibitors may be an effective therapeutic target for NSCLC treatment.

Keywords c-Met · HGF · Invasion · Non-small cell lung cancer · RhoA · MMP-2 · MMP-9 · TIMP-3

Abbreviations

HGF	Hepatocyte Growth Factor
NSCLC	Non-small cell lung cancer
MMP	Matrix metalloproteinases
TIMP	Tissue inhibitor of metalloproteinases
ECM	Extracellular matrix
TK	Tyrosine kinase
JM	Juxtamembrane
TM	Transmembrane
SNP	Single nucleotide polymorphism
IHC	Immunohistochemistry

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Introduction

Non-small cell lung cancer (NSCLC) accounts for 80–85% of primary lung cancer which is the leading cause of cancer mortality throughout the world [1]. Patients with NSCLC have a poor prognosis, despite recent advances in surgical, radiation, and medical treatments [2, 3]. Thus, molecular targeted therapy which offers a new therapeutic modality in this setting is being throughly investigated. Advances in the knowledge of molecular mechanisms of NSCLC have pointed out several molecular targets including the Hepatocyte Growth Factor (HGF)/c-Met signaling [4, 5]. Several mechanisms that lead to aberrant Met signalling in a variety of malignancies have been identified [4–11]. In some cancer cells, c-Met might be constitutively activated in a ligand-independent manner by gene mutation or overexpression with or without gene amplification.

It has been reported that c-Met mutations do not only cause tumor progression, but also increase the invasive and metastatic capacity of tumors [4, 12]. The majority of the activating mutations of c-Met reported previously are located within the sema domain, juxtamembrane (JM) and tyrosine kinase (TK) domains. However, in the lung cancer, the mutations of c-Met were predominantly present in nonkinase domain namely extracellular sema domain and intracellular juxtamembrane domain [13].

The juxtamembrane domain mutations of c-Met were shown to have activating phenotype with increased cell motility and tumorigenesis in small cell lung cancer [13]. Whereas, in NSCLC, role of c-Met mutations in activation of HGF/c-Met pathway is controversial. In the present study, Met receptor mutations were most commonly detected in the regions (coding Sema, JM and TK domains) investigated in NSCLC tissue samples.

Besides activating mutations, Met signalling pathway can be activated by overexpression of the kinase itself or its ligand HGF [14]. Overexpressions of both HGF and/or its receptor c-Met have been reported in NSCLC cell lines and patients [4–7]. It has also been shown that aberrations of HGF/c-Met signalling are involved in tumor cell motility, scattering, invasion and metastasis, suggesting to be a negative prognostic indicator for cell survival and recurrence [4, 7, 8].

Degradation or remodeling of the surrounding ECM, allows the tumor cells to migrate through the ECM tissue boundary. Cell motility is required for physiological processes of wound repair and organogenesis as well as for the pathologic process of tumor invasion [15]. HGF is known to induce cell migration in many types of cultured cells. Many studies have shown that HGF/c-Met signaling and mutationally activated Met increases the motility of epithelial and tumor cells [5, 15]. The Rho small G protein family members are signalling molecules downstream of HGF/c-Met and regulate cell adhesion and migration through reorganization of the actin cytoskeleton [15, 16]. Since, the RhoA is a key molecule of cell motility and is a downstream effector of HGF/c-Met pathway, we analyzed the level of RhoA expression and its correlation with HGF/c-Met expressions and clinicopathologic data.

The role of HGF/c-Met signaling in invasion and metastasis have been linked to their capability to increase proteases such as matrix metalloproteinases (MMPs) which lead to matrix degradation [9–11]. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that cleave components of the extracellular matrix (ECM) and basement membrane. Among the secreted MMPs, MMP-2 and MMP-9 are known to play key roles in tumor invasion and metastasis via their capacity to degrade type IV collagen, which is the major basement membrane component. MMP expression has been documented in several human cancers, including NSCLCs [17–20]. However, the clinical relevance of MMPs, especially that of MMP-9 and MMP-2 in NSCLC either remains controversial or unclarified [20–22].

ECM homeostasis is strictly maintained by a coordinated balance between the expression of MMPs and their spesific inhibitors, the TIMPs. The net balance between the TIMPs and MMPs generally correlates with tumorigenesis. However, studies have shown that the reduction in TIMP expression may actually increase with tumor progression [22–27]. Since the suggested role of HGF in invasion and metastasis of neoplastic cells has been linked to their capability to increase MMPs, while decreasing TIMPs**,** in this study, the correlations between expressions of HGF, c-Met, combined HGF/c-Met with those of MMPs (2, 9) and TIMPs (1, 3) were examined in NSCLCs.

Materials and Methods

Patient and Tissue Samples

The study was approved by the Review Board of Ethics of the Medical Faculty, Dokuz Eylül University, Izmir, Turkey. Paraffin-embedded tissue samples of 63 NSCLC patients were provided from Pathology archives of Dokuz Eylül University, School of Medicine. After diagnostic confirmation, these tissue blocks were used further for both DNA isolation and immunohistochemical (IHC) analysis. Clinical data of patients were obtained through a retrospective analysis of the reports in the Department of Medical Oncology. Tumour stages were classified according to the fifth edition of the TNM classification of the International Union Against Cancer (UICC) [28]. Additionally, nonneoplastic lung tissues were used as control.

Immunohistochemical Analysis

Sections (4 μm) were mounted poly-L-lysine coated slides from each representative tumor paraffin block. Sections (4 μm) were mounted poly-L-lysine coated slides from paraffin blocks for each representative tumor tissues as well as available normal lung tissues. All specimens were incubated with HGF-β, c-Met, MMP-9, RhoA (Santa Cruz, CA, USA), MMP-2 and TIMP-3 (NeoMarkers, Fremont, CA, USA) antibodies, using a standard avidin–biotin complex method. After deparafinization in xylene and rehydration with graded alcohols, sections were immersed in distilled water. For antigen masking, microwave treatment was performed in citric acid buffer, except for c-Met and MMP-9. For c-Met staining, proteinase-K pretreatment and for MMP-9 staining, a Tris–EDTA buffer, pH 8.0 were used. Primary antibodies were applied for 40 min at room temperature and linking antibody and streptavidin peroxidase complex (LSAB Kit, K-675; Dako, Carpinteria, CA) were added consecutively for 10 min at room temperature. Peroxidase activity was visualized with 0.03% 3, 3-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, MO). The sections were counterstained with Mayer's hematoxylene and mounted. Appropriate tissue sections which are known to react with antibody as a positive control for primary antibodies were also stained simultaneously. Negative controls were processed in the same manner after omitting the primary antibody treatment.

Evaluation of Immunostaining

Two independent pathologists (AK and ÇU) evaluated the staining pattern of the seven proteins separately and scored the protein expression in each the entire section. Strong staining was defined as staining clearly more intense than the staining of normal bronchial epithelium. The intensity of cytoplasmic or membranous staining was scored from 0 to 4+ (0: negative; 1+: weak; 2+: mild; 3+: moderate; 4+ intense staining). Extent of staining was scored from 0 to 4+ (0: negative; 1+: 0–25%; 2+: 26–50%; 3+: 51–75%; 4+: 76–100%) according to the percentage of the positively stained areas. The product of the intensity and extent of staining yielded final scores ranging between 0 and 16. Tumors with a final score less than or equal to 4 were considered as weakly positive (1+), and those with a final score greater than 4 were considered as strongly positive (2+) [29]. We further classified immunohistochemical staining intensity and extent of c-Met and other staining using three-scale scoring system, where 0, 1+ and 2+ equals to negative, weak and strong, respectively.

DNA Extraction and Polymerase Chain Reaction (PCR) Analysis

Paraffin sections (7 μm) were deparafinized in xylene and total DNA was extracted using Qiagen genomic DNA isolation kit (Qiagen, Valencia, CA, USA). PCR amplifications were performed using DNA Taq polymerase (MBI Fermentas GmBH, St-Leon-Rot, Germany) according to standart PCR techniques. The software program (Primer3 design) was used to design the optimal PCR primer sequences of c-Met. Primers were compared to the GenBank and EMBL nucleic acid sequence libraries using the BLAST program to ensure that they do not hybridize with any other known nucleic acid sequences under the conditions used. Since the span of exon 2 and exon 20-21 were longer than the expected range for the sequencing, these regions were amplified using three overlapping primer sets. The primer sequences and PCR profiles are given in Table 1.

Table 1 Primer sequences, PCR conditions and product sizes of sema, transmembrane and intracellular coding regions of c-Met

Exon No:	Forward primer (5′→3′)	Reverse Primer (5′→3′)	PCR products (bp)	Annealing temperature (°C)
2.1	TCTCTTCATTTCTGACAACTGAA	CCTGGCTGGGCTCTTCTATCT	580	55
2.2	CCCACAATCATACTGCTGACA	CCTTCCTTCTTTGTGGATCTCTT	499	55
2.3	AAATGCCTCTCTGGAGTGTATTC	GCACAAAAGAAGCCCTGGATA	416	55
13	GGACCCAAAGTGCTACAACC	GCACACAAGAATCGACGACA	322	65-58
14	GCCCATGATAGCCGTCTTTA	CAACAATGTCACAACCCACTG	256	65-58
15	TTCCTGTTTCAGTCCCCATTA	CACTGCTCTGTCAGTTGCTT	375	45
16	TGAAGCTCATAAAGGGTTTGA	AGGTTGCAAACCACAAAAGT	214	45
17	ATGCTAACTGTGTGGTTTACC	ATGGCTGGCTTACAGCTAGTT	269	65-58
18	GGCTTGAGCCATTAAGACCA	GCATTGAACAGTGGGAAACA	276	65-58
19	TCTATTTCAGCCACGGGTAAT	AGGAGAAACTAGAGATAACC	379	45
20-21 (1)	TGCCCAAAACAGAAACCGTATTG	GACCCTTTGAAGGCAGGCATT	326	65-58
20-21 (2)	GGGAGAAGACTCCTACAACCCGA	GTGTGGACTGTTGCTTTGACAT	405	65-58
20-21 (3)	TCCTTCTCTGTTGTCATCAGAAGA	CTGTGATCAAGAAGCCCTCAAT	423	43

Sequence Analysis

Direct sequencing was performed on an ABI-377 DNA Sequencer (Perkin Elmer Corp., Waltham, MA, USA) using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Sequencing reactions were performed in both directions to confirm any possible variations.

Statistical Analysis

The χ^2 test was used to analyze the associations between clinical and pathological determinants with molecular and immunohistochemical variables. Survival curves were calculated using Kaplan–Meier method and median survival times were compared with logrank test. Cox regression analysis was used to evaluate the effect of number of mutations on survival adjusted with radiotherapy and/or chemotherapy. A p value less than 0.05 was considered as significant. Data were analysed using SPSS for Windows version 11.0.

Results

Demographic Characteristic of Patients

Of the 63 NSCLC patients, 11 (18%) were stage I, 26 (41%) were stage II, 16 (25%) were stage IIIA, 7 (11%) were stage IIIB and 3 (5%) were stage IV. Their histopathological subtypes were as follows: 31 (49%) squamous cell carcinoma, 27 (43%) adenocarcinoma and 5 (8%) other histopatologic types (large cell, adenosquamous cell carcinoma) (Table 2).

Treatment

The treatment modalities applied to patients were categorized as surgical treatment, radiotherapy and chemotherapy. Of 63 patients, 60 (95.2%) were treated surgically [22 (34.9%) were treated by pneumonectomy, 38 (60.3%) were treated by lobectomy], 29 (46%) were treated by radiotherapy [25 (39.7%) were adjuvant after surgery, two (3.2%) were in combination with chemotherapy as sequential treatment following surgery and two (3.2%) were treated for palliative purposes]. Twelve patients (19%) had systemic chemotherapy [six (9.5%) were adjuvant chemotherapy, two cases (3.2%) were in combination with radiotherapy as sequential treatment following surgery, and two cases (3.2%) were neoadjuvant chemotherapy as sequential treatment following surgery and adjuvant radiotherapy, and two cases (3.2%) were treated for palliation as they were stage IV], and all the cases had six regimen of cisplatin plus gemcitabine.

Table 2 Demographic characteristics of the NSCLC patients

Parameter	n	%
Mean age (year)	63	58.2±9.9
Sex		
Female	7	11.1
Male	56	88.9
Stage		
Early	53	84.1
Late	10	15.9
TNM		
I	11	17.5
II	26	41.3
IIIA	16	25.4
IIIB	7	11.1
IV	3	4.8
Histological type		
Squamous	31	49.2
Adeno	27	42.9
Other *	5	7.9
Smoking		
Yes	55	87.3
No	8	12.7

*Other: includes adenosquamous cell carcinoma (n=2) and large cell carcinoma (n=3)

Correlations between HGF/c-Met Expressions and Clinicopathologic Parameters Lymph node invasion is involved with HGF/c-Met co-expression but not with HGF or c-Met expressions in NSCLC

c-Met expression, all cases (62/62 cases, 1 missing data) were positive with 81% (50/62) showing strong expression. The faint c-Met immunoreactivity was observed in bronchial epithelia, bronchiolar epithelia, and type II pneumocytes in normal lung tissues. Immunoreactivities were generally observed in the apical cytoplasm of these cells. Additionally, the staining intensity of c-Met in cancer tissues was stronger than those in surrounding noncancerous tissues.

For HGF, 95% (58/61) of NSCLC cases were positive with strong expression in 48% (29/61) of them. HGF/c-Met co-expression was found in 40% (24/60) of cases (Fig. 1). Neither HGF nor c-Met overexpression were correlated with age, smoking history, tumor size, histological subtype, stage, lymph node metastasis or relapse rate. However, the incidence of lymph node involvement was statistically higher in the cases with HGF/c-Met co-expression (p=0.040).

Correlations between HGF/c-Met Expressions and other Immunostainings The ratios of TIMP-3 and RhoA strong positive cases are high in positive cases for HGF expression or HGF/c-Met coexpression

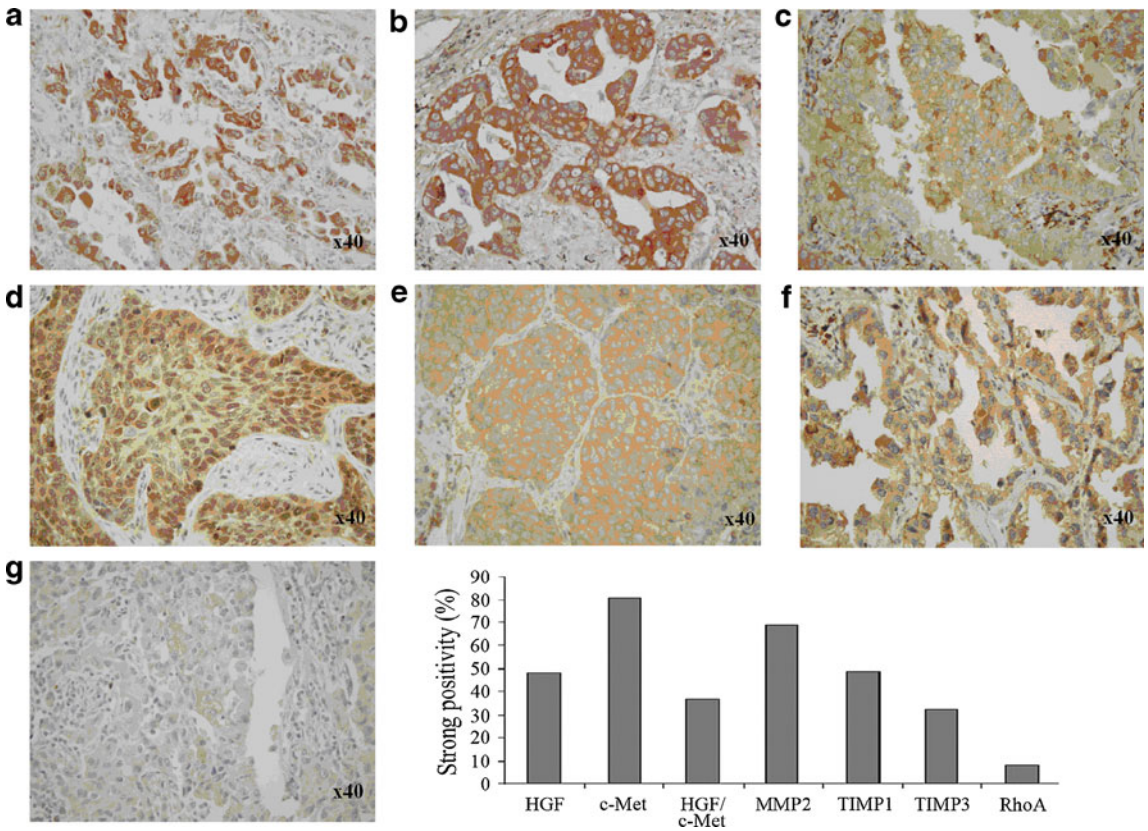

Fig. 1 Strong staining of HGF (**a**), c-Met (**b**), MMP-2 (**c**), TIMP-1 (**d**), TIMP-3 (**e**) and RhoA (**f**) in NSCLC tissues. MMP-9 strong expression was not encountered in any of the cases, weak positivity of MMP-9 was found in 11 of 60 (18%) cases (**g**). Percentage of strong positivity of immunhistochemical variables are shown in bar graphies in panel (**h**)

The strong positive immunostaining ratios for MMP-2, TIMP-1, TIMP-3, and RhoA were 69%, 49%, 32% and 8%, respectively (Fig. 1). MMP-9 strong expression was not encountered in any of the cases (Fig. 1).

In order to evaluate the relation of HGF and c-Met expressions in NSCLC with MMPs, TIMPs and RhoA, we assigned the cases into four groups, according to their HGF/c-Met expression patterns: Group 1 (both HGF and c-Met negative or weakly positive, n=7), Group 2 (both HGF and c-Met strongly positive, n=24), Group 3 (HGF negative or weakly positive, c-Met strongly positive, n=25), Group 4 (c-Met weakly positive, HGF strongly positive cases, n=4). The difference in the ratios of MMP-2, MMP-9, TIMP-1, TIMP-3 and RhoA positivity among these groups was investigated using χ^2 test. The ratios of TIMP-3 and RhoA positive cases were found to be higher in group 4 which had HGF overexpression (p=0.002 and p=0.045, respectively) and in group 2 which had HGF/c-Met overexpression (p=0.010 and p=0.011, respectively). The differences in the expression ratios of MMP-2, MMP-9 and TIMP-1 among each group were not statistically significant.

Correlations between Expressions of MMP-2, MMP-9, TIMP-1, TIMP-3, and RhoA and Clinicopathologic Parameters The strong expressions of MMP-2, TIMP-1 and RhoA do not correlate clinicopathologic parameters, except TIMP-3 which is expressed high in advanced stage NSCLC

The MMP-9 protein was weakly expressed in 11 out of 60 tumor samples (18%). MMP-9 strong expression was not encountered in any of the cases. Expectedly, no association between MMP-9 expression and clinicopathologic parameters. On the other hand, no correlation was found between the overexpressions of MMP-2, TIMP-1, TIMP-3, RhoA and the clinicopathologic parameters, except the association between TIMP-3 overexpression and the advanced stage NSCLC (Table 3, p=0.021). Besides, the expressions of HGF, c-Met, combined HGF/c-Met with those of MMPs (2, 9), TIMPs (1, 3) and RhoA were not significantly correlated with clinicopathologic parameters including age, smoking history, histological subtype, tumor size and relapse.

A Single Nucleotide Polymorphism (SNP) in Sema Domain and two SNPs in the Tyrosine Kinase Domain are Observed in c-Met Gene

The exon 2 and exons 13-21 of the *c-Met* gene were amplified by polymerase chain reaction (PCR) on DNA extracted from

Table 3 Association of HGF, c-Met, HGF/c-Met, MMP-2,-9, TIMP-1, -3, RhoA expressions and tumor stages in NSCLC

Stage	Early % n	Advanced % n
HGF	45 (23/51)	60 (6/10)
c-Met	81 (43/53)	78 (7/9)
HGF/c-Met	42 (21/50)	44 (4/9)
MMP-2	71 (36/51)	60 (6/10)
MMP-9	0 (0/51)	0 (0/10)
TIMP-1	51 (26/51)	40 (4/10)
TIMP-3	13 (17/52)	30 (3/10)*
RhoA	6 (3/51)	20 (2/10)

*p=0.021

NSCLC tumor tissues. Additionally, DNAs extracted from peripheral blood samples from healthy individuals and normal lung tissues were used as controls. c-Met genomic DNA bidirectional sequencing data did not show any alterations within exons 13-19. In sema domain of c-Met, a SNP in exon 2 (rs11762213, G to A transition at the third positions of codon 331) was observed in one NSCLC tissue.

Two synonymous SNPs were observed in exon 20-21 encoding tyrosine kinase domain of c-Met receptor (Fig. 2). First alteration was C to T transition (rs41736) and found in 26% (11 of 43) of the tumor tissue. This transition was not observed any of the control tissues and blood samples. The second one was G to A transition (rs2023748) and found in 14% (6 of 42) of the tumor tissues. This transition also observed in both normal lung tissues and blood samples. All observed nucleotide alterations of c-Met gene we have identified are present in the National Center for Biotechnology Information Entrez single nucleotide polymorphism database search.

The presence of nucleotide alterations in exon 2 and exon 20-21 of c-Met gene were not significantly correlated with c-Met protein expressions. Additionally, there was no statistically significant correlation between the presence nucleotide alterations of c-Met and clinicopathologic parameters.

The Effects of Expressions of HGF, c-Met, MMP-2, MMP-9, TIMP-1, TIMP-3, RhoA and c-Met Gene Alterations on Survival: RhoA Overexpression and Double Nucleotide Alterations in c-Met Decrease Survival Time

Mean survival rate was 33.6±24.0 (95% confidence interval: 27.55–39.65) (3–102 months), and mean disease-free survival rate of all groups was 30.4±23.2 (95% confidence interval: 52.4–89.5). One-, 2- and 3-year survival rates were 65%, 46% and 30%, respectively. There was no statistical significance between HGF, c-Met, HGF/c-Met, MMPs, TIMPs and survival. However, survival was found to be significantly lower in the cases with RhoA overexpression (log-rank test, p<0.05, Fig. 3).

There was no statistically significant association between nucleotide alteration in exon 2 (sema domain) and survival. While C to T or G to A transitions in exon 20-21 of c-Met (TK domain) were not particularly related to survival, surprisingly copresence of the both C to T and G to A transitions in the same case significantly decreased survival time (log-rank test, p=0.005, Fig. 3).

Patients with double SNP in TK domain had a 4.6-fold higher risk of death than those with single SNP or lack of

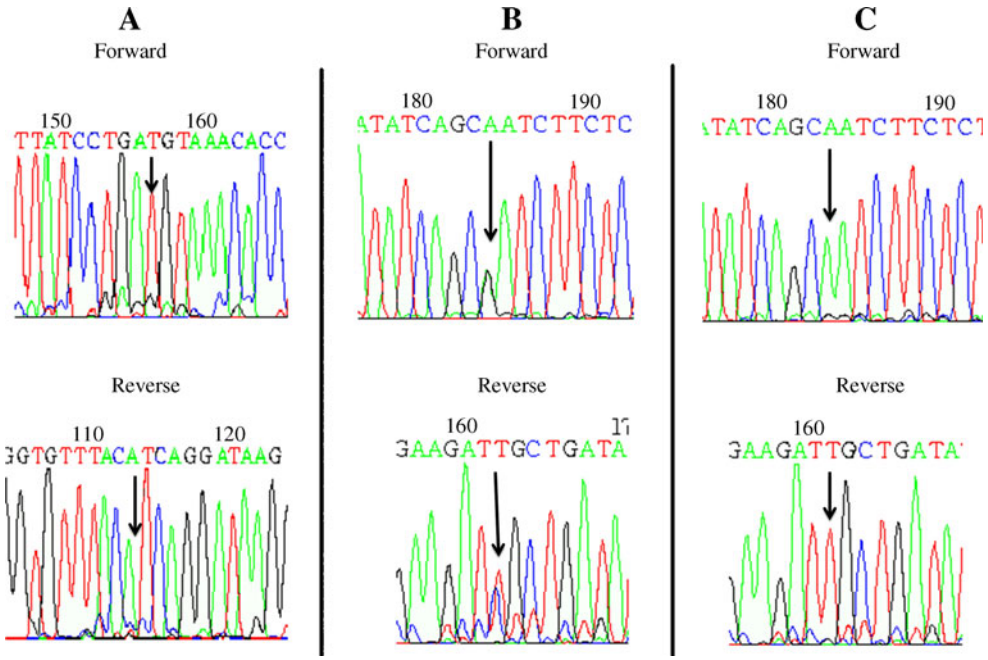

Fig. 2 Two SNPs in TK domain of c-Met gDNA identified in NSCLC. The SNP in panel (**a**), c1286 T–>C transition (rs41736) was homozygous, that in panel (**b**) and (**c**), g1339 A–>G transition (rs2023748) heterozygous (**b**) and homozygous (**c**) identified in exon 20-21 of c-Met. Both SNPs (rs41736 and rs2023748) were found in 6 cases

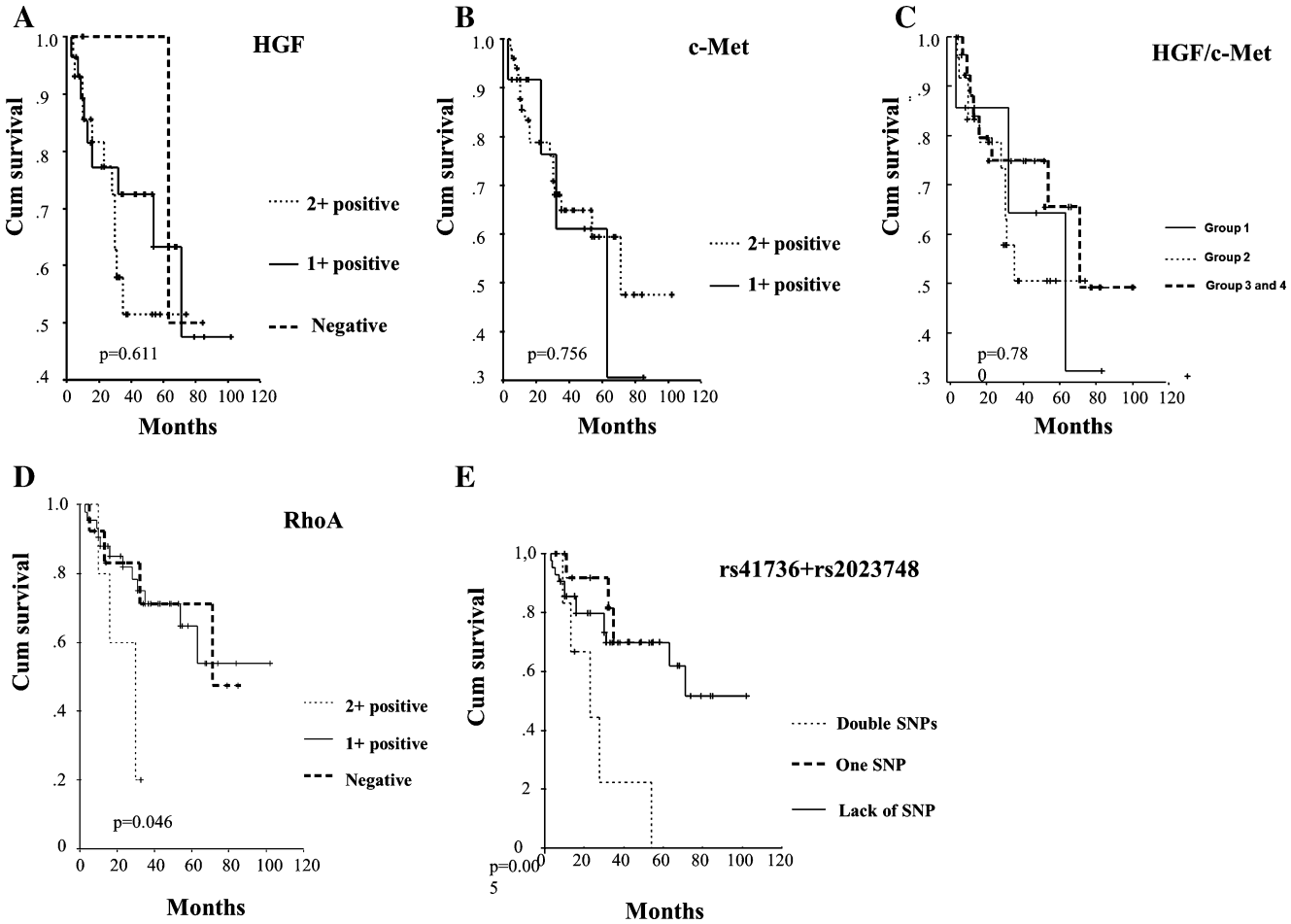

Fig. 3 Kaplan-Meier survival curves established for patients with NSCLC based on strong positivity (2+) compared to cases with negative or weak positivity of HGF, c-Met, HGF/c-Met and RhoA (**a-d**). The cases with RhoA overexpression were found to have decreased survival time (p=0.046). Cumulative survival in patients with double SNPs (rs41736 and rs2023748) compared to patients with one SNP (rs41736 or rs2023748) or lack of SNP **(e)**. While one SNP (rs41736 or rs2023748) in c-Met were not related to survival individually, those cases with double SNPs (rs41736 and rs2023748) had a statistically significant lower survival (p=0.005). Curves were compared with log-rank test

SNP (hazard ratio (HR): 4.63, 95% confidence interval, 1.64–13.12, p=0.004), and this association remained significant in Cox regression analysis adjusted for surgery, radiotherapy or chemotherapy treatment (HR): 4.12, 95% confidence interval, 1.40–12.13, p=0.01). There were no differences in the treatment regimens between patients with double SNP single SNP or lack of SNP and (p>0.05).

Discussion

In this study, we observed HGF and c-Met overexpressions (48% and 81%, respectively) in NSCLC tissues. It has been published that in NSCLC tissue, c-Met activation may take place either ligand-dependently or ligand-independently via c-Met overexpression [5, 6, 30]. Since c-Met overexpressing cases (81%) were found to be higher than that of HGF overexpressing cases (48%), it is possible that **c**-Met might be activated in a HGF-independent manner by overexpression of c-Met in those cases.

One of the mechanism underlying ligand independent c-Met activation is presence of receptor mutations. Due to the increasing knowledge in the role of missense mutations as an activating factor of c-Met; we planned to investigate the activating mutations in the sema domain, juxtamembrane (JM) and tyrosine kinase (TK) domains of c-Met. There are limited studies about the role of c-Met mutations in the activation of HGF/c-Met pathway in NSCLC. Ma et al. [13] published that Sema and JM domain mutations of c-Met may have an activating role in the progression SCLC via increasing cell motility. Same researchers also reported the presence of Sema domain mutations in NSCLC cell lines [4]. However, we didn't observe any amino acid alterations within exons 2 encoding the sema domain and exons 13-21 encoding the transmembrane (TM), JM and TK domains of c-Met in

NSCLC tissues. When we analyzed the relation between nucleotide alterations in c-Met gene with clinicopathological parameters, we found no correlation except shorter survival time. It has been published that silent mutations or SNPs in a protein may decrease mRNA level or alters the substrate specificity of protein by changing cotranslational folding [31, 32]. Kimchi-Sarfaty et al. [32] reported that when an SNP or silent mutation changes frequent codons with rare codons the timing of cotranslational folding affected, and it may results in altered function.

Since the presence of two SNPs that cause rare codon usage in tyrosine kinase domain of c-Met are correlated with shorter survival time in our study, these alterations may change cotranslational folding of c-Met that modify its interaction with downstream effectors.

Nevertheless, our results suggest that c-Met mutations are not a common phenotype in NSCLC and there is little impact on activation of c-Met. This data is consistent with the previous publications on the deregulation of Met activity in other thoracic malignancies and small cell lung cancer [12–14]. They suggested that c-Met activity has been linked and attributed mainly to overexpression, however the downstream targets of HGF/cMet signaling which may contribute to tumor progression is not focused in these studies. Similar to previous studies, the incidence of lymph node invasion was found statistically higher in the cases with HGF/c-Met co-expression in our study. It has been known that one of the most important processes that determine the invasion and metastasis capacity of tumor cells is the acquisition of cell motility. It has been known that RhoGTPases increase cell motility, and HGF promotes RhoA-mediated cell motility [5, 15, 16]. It has also been reported that the small GTP binding proteins Rho and Rac are required for HGF-induced scatter and prior cytoskeletal rearrangements [16]. We found a statistically significant relation between the increased HGF and/or c-Met expressions and increased RhoA expression was found in NSCLC tissues. Therefore, the lymph node invasion induced by HGF/c-Met coexpression is thought to be mediated by RhoA overexpression, in our study. Additionally, the cases with RhoA overexpression were found to have significantly lower survival time. Taken together, these data indicated that the presence HGF and/or c-Met overexpression along with RhoA overexpression might be specific indicator for poor survival in patients with NSCLC. Our results are correlated with the data of Li et al. [33] showing that the prognostic relevance of overexpression of RhoA and shorter survival in hepatocellular carcinomas. Similar with Li et al.'s results, we observed a negative correlation with RhoA expression and survival time, we found significantly lower survival time in the cases with RhoA overexpression.

Another important step which has an effect on invasion capacity of tumor cells is ECM degradation. It has been reported in numerous studies that the expressions of MMP-2 and MMP-9, which degrade collagen IV, a major component of ECM, increase in many carcinomas. The importance of HGF and/or c-Met overexpression in the prognosis of NSCLC might be linked to their capability to increase MMPs expressions. Although, the role of MMPs in NSCLC has been reported in several studies, in our knowledge, there is no other study focused on the relation between HGF/c-Met and MMPs in the progression of NSCLC. Similar with the study published by Thomas et al. [23], we found strong MMP-2 expression in 69% of cases. MMP-9 was found to be either weakly expressed (11 cases) or not expressed (52 cases) similarly with Nawrocki et al. [34]. No correlation was obtained between HGF/c-Met overexpression and MMP-2 immunoreactivity in our study.

Since the loss of balance between MMPs and TIMPs is expected to lead an increase in the invasion capacity of tumor cells, the lack of any relation between HGF/c-Met and MMPs prompted us to investigate the relation of HGF/c-Met with TIMPs. We have chosen TIMP-1 and TIMP-3 that are related with HGF/c-Met pathway and also related to NSCLC. In our study, TIMP-1 and TIMP-3 overexpressions were found in 49% and 33% of the cases, respectively. Aljada et al. [26] reported that 27% of NSCLC cases expressed TIMP-1 and its expression was not correlated with MMP-2 nor MMP-9. Similar with Aljada's results, no relation was found between TIMP-1 and either MMP-2 or MMP-9 in our study. Mino et al. [27] reported TIMP-3 expression in 22.4% of NSCLCs and it was found to be inversely related to MMP-2 expression. Although the ratio of TIMP-3 expressing cases (33%) was similar to the previous reports, no relation between TIMP-3 and MMP-2 or MMP-9 was found in our study. Interestingly, while TIMP-1 expression was not found to be related to either HGF or c- Met expressions or their co-expressions, TIMP-3 expression was statistically higher in those cases with HGF overexpression ($p=0.002$) and HGF/c-Met co-expression ($p=0.010$). Castagnino et al. [35] reported that HGF transiently induced TIMP-3 mRNA and increased TIMP-3 protein secretion in keratinocytes, as well as kidney and mammary epithelial cells. The possibility of HGF induction leading to sustained TIMP-3 secretion in neoplastic tissues remains to be investigated.

The reported results of studies regarding the relations of HGF/c-Met, MMPs and TIMPs with clinicopathological parameters, including tumor's stage and patient's survival in NSCLCs have been controversial [25–27, 36]. In accordance with some, and contrary to other studies, in this study, expressions of HGF/c-Met, MMP-2, -9 and TIMP-1 were not found to be related to either clinicopathological parameters or survival. However, although it was not related to survival, TIMP-3 expression was found to be higher in advanced stage NSCLCs ($p=0.04$). In a variety of

experimental cancers, TIMP-3 expression was shown to suppress primary tumor growth, angiogenesis, invasion and metastasis [37–39]. It was also reported that it leads to oncogenic transformation of fibroblasts in cell culture [40]. In addition, it has been suggested to play a role in progression of uveal melanoma [41]. It appears that the role of TIMP-3 in progression of NSCLCs needs further investigation.

Our results suggest that the implicated role of HGF/c-Met in lymph node invasion through the degradation of ECM is probably not related either to their suggested effect in increasing the expression of MMPs (2 and 9) or decreasing the TIMP-1 expression. Instead, we found an association of HGF overexpression or HGF/c-Met co-expression with RhoA and TIMP-3 overexpressions, an increased TIMP-3 expression in advanced stage and association of RhoA overexpression with poor survival in NSCLC. These results suggest that HGF and HGF/c-Met might exert their effects in tumor progression in association with RhoA and probably with TIMP-3. And also, these data indicated that the presence HGF/c-Met overexpression along with RhoA or TIMP-3 overexpression may be poor prognostic factor in patients with NSCLC.

In conclusion, the blockage of HGF/c-Met pathway in combination with RhoA and/or the TIMP-3 inhibitors may be an effective target for NSCLC treatment.

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