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DNA copy number gains in malignant pleural mesothelioma

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Abstract. Malignant pleural mesothelioma (MPM) is a highly aggressive tumor with an extremely poor prognosis. The incidence of MPM is increasing as a result of widespread exposure to asbestos. The molecular pathogenesis of MPM remains unclear. The present study analyzed the frequency of various genomic copy number gains (CNGs) in MPM using reverse transcription-quantitative polymerase chain reaction. A total of 83 primary MPMs and 53 primary lung adenocarcinomas were analyzed to compare the CNGs of *EGFR*, *KRAS*, *MET*, *FGFR1* and *SOX2*. In MPM, the CNGs of *EGFR*, *KRAS*, *MET*, *FGFR1* and *SOX2* were detected in 12 (14.5%), 8 (9.6%), 5 (6.0%), 4 (4.8%) and 1 (1.2%) of the samples, respectively. In lung adenocarcinomas, the CNGs of *EGFR*, *KRAS*, *MET*, *FGFR1* and *SOX2* were detected in 21 (39.6%), 12 (22.6%), 5 (9.4%), 10 (18.9%) and 0 (0.0%) of the samples, respectively. The CNGs of *EGFR*, *KRAS* and *FGFR1* were significantly less frequent in the MPMs compared with the lung adenocarcinomas (P=0.0018, 0.048 and 0.018, respectively). Overall, the MPMs exhibited these CNGs less frequently compared with the lung adenocarcinomas (P=0.0002). The differences in CNGs between the two tumor types suggested that they are genetically different.

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Introduction

Malignant pleural mesothelioma (MPM) is a tumor derived from the mesothelial cells lining the pleural spaces. MPM has highly invasive and aggressive clinical characteristics. Approximately 80% of MPM patients have a history of occupational asbestos exposure, which is considered to be a risk factor for the development of the disease (1). The molecular pathogenesis of MPM is not well understood. The most common mutations in MPMs are losses in 9p21, 1p36, 14q32 and 22q12, and gains in 5p, 7p and 8q24, which have been detected by comparative genomic hybridization analysis (2,3). Homozygous deletion of the 9p21 locus encoding two critical cyclin-dependent kinase inhibitors, $p16^{INK4a}$ and $p15^{INK4b}$, have been reported in up to 80% of MPMs, and this mutation may be of diagnostic utility (4,5). The tumor suppressor neurofibromin 2 is encoded by the *NF2* gene, located on chromosome 22q12. Mutations in *NF2* are found in ~40% of MPMs, and heterozygous loss of *NF2* is identified in ~74% of MPMs (6,7). Mutations are rare in the *TP53* and *RAS* genes, which are frequently present in epithelial solid tumors (8,9). Epigenetic alterations, such as DNA methylation, have been found in MPMs, which have a different profile compared with lung cancer (10-12). MPMs, particularly of the epithelioid subtype, may be hard to differentiate from adenocarcinoma arising in the lung periphery, and epidemiological evidence indicates that asbestos and smoking are shared risk factors for these diseases (2,13,14). Currently, the differential diagnosis of MM is based on a range of morphological analyses, including a combination of histological and immunohistochemical staining, and electron microscopy (13,15,16).

Cytogenetic studies have been performed on MPMs and adenocarcinomas arising in the lung periphery, however, no chromosomal aberrations specific to either of the tumor types have been identified (2,14).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is a method for evaluating DNA copy number

changes, including losses, gains and amplifications of DNA sequences (17-19). Copy number gains (CNGs) of *EGFR* and *KRAS* have been observed in lung cancer, particularly in adenocarcinoma (18,20). Furthermore, CNGs of *FGFR1* and *SOX2* have been observed in lung cancer, particularly in squamous cell carcinoma (21-25). c-Met was recently reported to be activated in MPM by overexpression or mutations in *MET* (26), and *MET* amplification is a known cause of resistance to *EGFR*-tyrosine kinase inhibitor (TKI) treatment in lung cancer (27). RT-qPCR was used in the present study on 83 primary MPM and 53 primary lung adenocarcinomas to compare the CNGs of *EGFR*, *KRAS*, *MET, FGFR1* and *SOX2*.

Materials and methods

Tumor samples. Surgically resected specimens of 53 lung adenocarcinomas and 83 MPMs (57 epithelioid, 8 sarcomatoid, 15 biphasic, 2 desmoplastic and 1 lymphohistiocytic) were obtained. All the lung adenocarcinomas and 11 of the MPM samples were obtained from Okayama University Hospital (Okayama, Japan). Another 18 MPMs were obtained from Yamaguchi-Ube Medical Center (Ube, Japan), 2 were obtained from Okayama Rosai Hospital (Okayama, Japan) and the remaining 52 were obtained from Karmanos Cancer Center (Detroit, MI, USA). All Japanese samples were collected between March 2002 and September 2011, and all samples from the USA were collected >10 years ago. Resected tumors were stored at -80˚C until DNA extraction. Permission from the Institutional Review Board and informed consent were obtained at each collection site.

DNA extraction. Genomic DNA was obtained from primary tumors by standard phenol:chloroform (1:1) extraction, followed by ethanol precipitation, or using a DNeasy Tissue kit (Qiagen, Inc., Valencia, CA, USA).

RT-qPCR for copy number evaluation. CNGs of *EGFR*, *KRAS, MET, FGFR1* and *SOX2* genes were determined by RT-qPCR assays using Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), as previously described (18,19). Briefly, samples of 1 μl were analyzed per assay using with StepOne Plus Real-Time PCR System (Themo Fisher Scientific). PCR conditions were initial denaturation at 95˚C for 10 min followed by 40 cycles of amplification at 95˚C for 15 sec and 60˚C for 60 sec. The samples were analyzed in triplicate using StepOne Plus RT PCR software (version 2.0; Themo Fisher Scientific) and the *LINE1* gene was used as a reference gene for all copy number analyses, as this is the most abundant autonomous retrotransposon in the human genome, constituting 17%. Each amplification reaction was checked for the absence of non-specific PCR products by performing a melting curve analysis. The copy number calculation was conducted using the comparative cycle threshold (Ct) method following validation of the PCR reaction efficiency of *EGFR, KRAS, MET, FGFR1, SOX2* and *LINE1*. The PCR primer sequences for *EGFR, KRAS, MET* and *LINE1* primers have previously been described (17-19). The PCR primer sequences for *FGFR1* and *SOX2* were designed by Primer 3 plus software and by modification of the sequences. The PCR primer sequences were as follows: *FGFR1* forward, 5'-AGC CAC CAC ATG GCA TAC TT-3' and reverse, 5'-GGT GAC AAG GCT CCA CAT CT-3'; and *SOX2* forward, 5'-CGT CAC ATG GAT GGT TGT CT-3' and reverse, 5'-GCC GCC GAT GAT TGT TAT TA-3'. The relative copy number of each sample was determined by comparing the ratio of the target gene to *LINE1* in each sample with the ratio of these genes in normal human genomic DNA (EMD Biosciences, Darmstadt, Germany) prepared from a mixture of human blood cells from 6-8 donors, as a diploid control. Our previous study defined a copy number of ≥4 as a gene gain in cell lines (17,18). However, considering the contamination by non-malignant cells in primary samples (estimated mean per tumor, 50% tumor cells and 50% non-malignant cells), the cut-off value of 3 copy numbers rather than 4 was used for primary tumors in this study (17).

Detection of EGFR mutations. The *EGFR* mutational status was determined using a PCR-based length polymorphism and restriction fragment length polymorphism assay, as previously described (28). Briefly, the common deletions of exon 19 were distinguished from the wild-type based on PCR product length polymorphisms using 12% polyacrylamide gel electrophoresis (PAGE) and ethidium bromide staining. For the exon 21 L858R mutation, *Sau*96I digestion, which specifically digests the mutant type, was performed prior to 12% PAGE.

Statistical analyses. Differences between the two groups were assessed using the χ^2 test or Fisher's exact test as required. All data were analyzed using JMP software version 9.0.0 (SAS Institute Inc., Cary, NC, USA). For all analyses, $P<0.05$ was considered to indicate a statistically significant difference.

Results

CNGs in MPMs and lung adenocarcinomas. In the 83 MPM samples, the CNGs of *EGFR*, *KRAS, MET, FGFR1* and *SOX2* were detected in 12 (14.5%), 8 (9.6%), 5 (6.0%), 4 (4.8%), and 1 (1.2%) of the samples, respectively. In the epithelioid subtype of MPM (n=57), the CNGs of *EGFR*, *KRAS*, *MET*, *FGFR1* and *SOX2* were detected in 7 (12.3%), 5 (8.8%), 3 (5.3%), 4 (7.0%) and 0 (0.0%) of the samples, respectively. In the other subtypes of MPMs (n=26), the CNGs of *EGFR*, *KRAS*, *MET*, FGFR and *SOX2* were detected in 5 (19.2%), 3 (11.5%), 2 (7.7%), 0 (0%) and 1 (3.8%) of the samples, respectively. In the 53 lung adenocarcinomas, the CNGs of *EGFR*, *KRAS*, *MET*, *FGFR1* and *SOX2* were detected in 21 (39.6%), 12 (22.6%), 5 (9.4%), 10 (18.9%) and 0 (0.0%) of the samples, respectively (Table I; Fig. 1). Three cases of MPMs were demonstrated to have numerous CNGs of *EGFR* (269, 62 and 14, respectively). The CNGs of *EGFR*, *KRAS* and *FGFR1* were significantly less frequent in the MPMs compared with the lung adenocarcinomas (P=0.0018, 0.048 and 0.018, respectively). In the epithelioid subtype of MPMs, the CNGs of *EGFR* were significantly less frequent than those in the lung adenocarcinomas (P=0.0018), and in other subtypes of MPMs, the CNGs of *FGFR1* were significantly less frequent compared with those of the lung adenocarcinomas (P=0.026). In the MPMs, an absence and presence of CNGs were observed in 64 (77.1%) and 19 (22.9%) of the 83 cases, respectively. In the epithelioid MPMs, absent/present CNGs were observed in 47 (82.5%) and 10 (17.5%) of the 57 cases, respectively. In the other subtypes of

Table I. CNGs of *EGFR*, *KRAS*, *MET*, *FGFR1* and *SOX2* in MPMs and lung adenocarcinomas.

	MPMs (n=83)							
	All (n=83)		Epithelioid subtype (n=57)		Other subtypes (n=26)		Lung adenocarcinoma (n=53)	
Genes	No.	%	No.	%	No.	%	No.	%
EGFR	12[a]	14.5	7[a]	12.3	5	19.2	21	39.6
KRAS	8[b]	9.6	5	8.8	3	11.5	12	22.6
MET	5	6.0	3	5.3	2	7.7	5	9.4
FGFR1	4[b]	4.8	4	7.0	0[b]	0.0	10	18.9
SOX2	1	1.2	0	0.0	1	3.8	0	0.0

CNGs of *FGFR1* and *KRAS* were significantly less frequent in MPMs compared with lung adenocarcinomas. In epithelioid MPMs, CNGs of *EGFR* were found to be significantly less frequent compared with lung adenocarcinomas. In other types of MPMs, CNGs of *FGFR1* were found to be significantly less frequent compared with lung adenocarcinomas ([a]P<0.05; [b]P<0.01). CNGs, copy number gains; MPMs, malignant pleural mesotheliomas.

Table II. Frequency of the absence or presence of CNGs in MPMs and lung adenocarcinomas.

	Absence of CNGs		Presence of CNGs	
Cancer type	No.	%	No.	%
Malignant pleural mesothelioma (n=83)[a]	64[a]	77.1	19	22.9
Epithelioid subtype (n=57)[a]	47[a]	82.5	10	17.5
Other subtypes (n=26)	17	65.4	9	34.6
Lung adenocarcinoma (n=53)	24	45.3	29	54.7

Frequency of none of CNGs in MPMs and epithelioid MPMs was significantly higher compared with lung adenocarcinomas ([a]P<0.01). CNGs, copy number gains; MPM, malignant pleural mesothelioma.

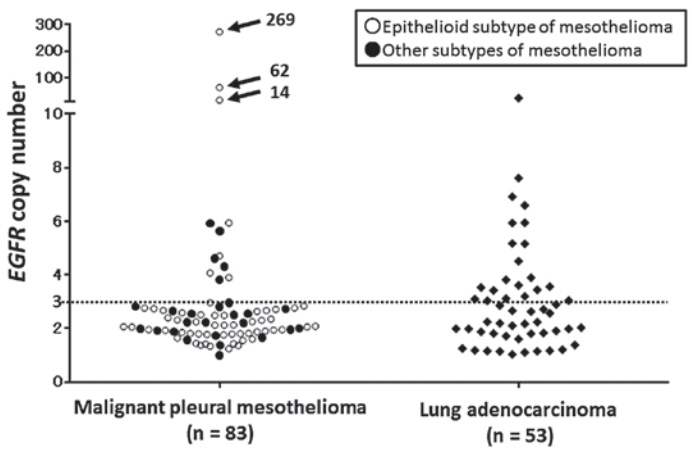

Figure 1. *EGFR* gene copy number, determined by reverse transcription-quantitative polymerase chain reaction in malignant pleural mesotheliomas (MPMs) and lung adenocarcinomas. Copy numbers >3 were considered as copy number gain (CNG). Three cases of MPMs were shown to have high CNGs of *EGFR* (269, 62 and 14, respectively).

the MPMs, the absence and presence of CNGs were observed in 17 (65.4%) and 9 (34.6%) of the 26 cases, respectively. In the lung adenocarcinomas, the absence and presence of CNGs were observed in 24 (45.3%) and 29 (54.7%) of the 53 cases, respectively (Table II). The MPMs and the epithelioid subtypes of the MPMs had less frequent CNGs than the lung adenocarcinomas (P=0.0002 and P=0.0001, respectively).

EGFR mutations. No *EGFR* mutation was detected in the 83 MPMs. In the lung adenocarcinomas, *EGFR* mutations

were detected in 21 (39.6%) cases; 14 cases exhibited an exon 19 deletion and 7 cases exhibited an exon 21 mutation (L858R).

Discussion

The main finding of the present study is that the pattern of DNA CNGs of MPM is different from that in lung adenocarcinoma. MPMs exhibited less CNGs of the genes examined in compared with the lung adenocarcinomas. The epithelioid subtype of MPM, which is often difficult to distinguish from lung adenocarcinoma, similarly exhibited these CNGs less frequently compared with the lung adenocarcinomas. To the best of our knowledge, only a limited number of studies have previously analyzed the presence and frequency of *EGFR* CNGs in MPMs (2,29-32), and no studies have focused on CNGs of *KRAS*, *MET*, *FGFR1* or *SOX2* in MPM. A large number of samples (n=83) were screened in the present study, whereas the previous studies were based on smaller sample sizes and may have underestimated the true frequency of such CNGs.

Although CNGs of *SOX2* were seldom observed in the MPMs and lung adenocarcinomas, the CNGs of the remaining four genes were detected in the MPM samples to a certain extent. The fact that the CNGs of four genes in the MPMs were less frequent in comparison to the lung adenocarcinomas suggested that CNG may not be a pivotal mechanism for the activation of oncogenes in MPMs, and that different mechanisms may be of greater importance. It has been previously reported that *EGFR* is overexpressed in 60-70% of MPM tissue specimens; however, it is not overexpressed in the normal mesothelium (29,33). Furthermore, exposure to asbestos fibers is known to cause *EGFR* aggregation (34). In the present study, *EGFR,* located at 7p12-p13, was the most frequent gene to exhibit CNGs (12 out of 83 MPMs and 20 out of 53 lung adenocarcinomas). Bjorkqvist *et al* (2) reported similar results, such as gains of genetic material in 5p, 6p and 7p between MPMs and lung adenocarcinomas. The study detected a gain in 7p in 7 out of 34 MPMs and 11 out of 30 lung adenocarcinomas (2). MPMs rarely harbor *EGFR* mutations (31,35-37). There were no *EGFR* mutations detected in MPMs in the present study, as expected. Upon analysis, three cases of MPMs exhibited high *EGFR* gene amplification (CNG>10), and these cases were all epithelioid MPMs, which was consistent with the previous studies by Okuda *et al* (29) and Enomoto *et al* (31). It remains unclear whether high-level amplification of *EGFR* is more prominent in MPMs compared with lung adenocarcinomas, although the frequency of CNGs for *EGFR* is lower in MPMs compared with lung adenocarcinomas. In MPMs with *EGFR* amplification, the inhibition of *EGFR* pathways should exert an antitumor effect. In lung cancer, the results of two randomized phase III trials that compared a placebo to erlotinib or gefitinib treatment indicated that *EGFR* copy number detected by fluorescence *in situ* hybridization was the best predictor of survival (38). Patients with colorectal cancer who responded to anti-*EGFR* treatment with cetuximab or panitumumab exhibited an increased *EGFR* copy number (39). Although two phase II studies of single-agent EGFR-TKI therapy to treat MPMs failed to demonstrate their clinical efficacy, in the gefitinib trial, 2 of 43 MPM patients responded to gefitinib (40,41). These data suggest that a small proportion of patients (with *EGFR* gene amplification) may be candidates for anti-*EGFR* treatment (29).

In conclusion, the present study detected novel CNGs in genes other than *EGFR*. MPM samples exhibited these CNGs less frequently compared with lung adenocarcinomas. The differences in DNA CNG between the two tumor types suggested that they are genetically different.

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