Mutation analysis of the EGFR gene and downstream signalling pathway in histologic samples of malignant pleural mesothelioma

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Background: As epidermal growth factor receptor (EGFR) is involved in the pathogenesis of malignant pleural mesotheliomas (MPMs), the anti-EGFR drugs may be effective in treating MPM patients. Mutations of the EGFR gene or its downstream effectors may cause constitutive activation leading to cell proliferation, and the inhibition of apoptosis and metastases. Consequently, molecular profiling is essential for select patients with MPM who may respond to anti-EGFR therapies.

Methods: After manual macrodissection, genomic DNA was extracted from 77 histological samples of MPM: 59 epithelioid, 10 biphasic, and 8 sarcomatoid. Epidermal growth factor receptor gene mutations were sought by means of real-time polymerase chain reaction (PCR) and direct sequencing, KRAS gene mutations by mutant-enriched PCR, and PIK3CA and BRAF gene mutations by direct sequencing.

Results: Gene mutations were identified in nine cases (12%): five KRAS, three BRAF, and one PI3KCA mutation; no EGFR gene mutations were detected. There was no difference in disease-specific survival between the patients with or without gene mutations (P = 0.552).

Conclusions: Mutations in EGFR downstream pathways are not rare in MPM. Although none of those found in this study seemed to be prognostically significant, they may support a more specific selection of patients for future trials.

Malignant mesotheliomas are relatively rare, highly malignant tumours that arise from the mesothelial cells lining the serosal cavities of the body. The most frequent are malignant pleural mesotheliomas (MPMs) (Boutin et al., 1998), whose estimated annual incidence in Europe (15–33 cases per million inhabitants) is expected to increase further over the next 20 years because of their long period of latency (Peto et al., 1999). The main carcinogen associated with MPMs is asbestos, and occupational exposure to it has been considered the main risk factor (Wagner et al., 1960; Lanphear and Buncher, 1992; Carbone et al., 2002); however, other potential carcinogenic agents include infection by Simian Virus 40 and radiation exposure (Yang et al., 2008).

The median survival of patients with MPM is currently 12 months from the time of diagnosis despite treatment (Vogelzang et al., 2003). The European Organisation for Research and Treatment of Cancer has indicated that the main predictors of a negative prognosis are a poor performance status (PS), high white blood cell counts, male gender, a sarcomatoid subtype, anaemia,
and thrombocytosis (Curran et al., 1998), but it has been recently suggested that short survival may also be related to a high nuclear tumour grade, a high MIB-1 labelling index (Comin et al., 2000), and high epidermal growth factor receptor (EGFR) expression in tumour cells (Rena et al., 2011).

There is no standard of care for MPM, but systemic chemotherapy is the only possible treatment option for most patients at the time of diagnosis. It has been found that pemetrexed combined with cisplatin is more effective than platinum alone in terms of overall survival (12.1 vs 9.3 months), time to progression (5.7 vs 3.9 months), and objective responses (41.3% vs 16.7% of tumour shrinkage) (Vogelzang et al., 2003), but, as most patients progress during or shortly after these first-line treatments, there is a clear need to develop more effective antitumour agents.

Epidermal growth factor receptor is a receptor tyrosine kinase that is overexpressed in 60–70% of MPM tissue specimens (mainly of the epithelial subtype), but not in the normal mesothelium (Dazzi et al., 1990; Destro et al., 2006; Okuda et al., 2008). Exposure to asbestos fibres causes EGFR aggregation, and the subsequent autophosphorylation and activation of EGFR activates both the RAS/RAF/MAPK pathway (which induces cell proliferation, metastasis, and invasion) (Pache et al., 1998) and the PI3KCA/PI3K/mTOR pathway, which leads to the inhibition of apoptosis (Suzuki et al., 2009). Consequently, inhibiting EGFR pathways should have an antitumoral effect. Two classes of EGFR inhibitors have been developed for cancer therapy: tyrosine kinase inhibitors (TKIs), which block EGFR autophosphorylation by competing with ATP binding, and anti-EGFR monoclonal antibodies (mABs), which compete with the ligand binding the extracellular domain of EGFR. It has been reported that mutation analysis of the EGFR gene and of some of its downstream signal-transduction proteins predicts the response of lung adenocarcinomas to TKIs (Shepherd et al., 2005) and the response of colorectal cancer to anti-EGFR mABs (cetuximab, panitumumab) (Jonker et al., 2007).

Preclinical studies have shown that EGFR TKIs are highly efficacious (Barbieri et al., 2011), but two phase II studies of gefitinib and erlotinib used alone to treat malignant pleural and peritoneal mesotheliomas failed to demonstrate their clinical efficacy, although it needs to be pointed out that the patients in both trials were not selected on the basis of any molecular criteria (Giovannia et al., 2005; Garland et al., 2007). One recent study has shown that cetuximab effectively blocks the growth of MPM cells in cell cultures and mouse models (Kurai et al., 2012) and, as in the case of colorectal cancer and lung adenocarcinomas, the potential efficacy of these TKIs in MPM may depend on the mutation status of EGFR gene and its downstream effectors (Lièvre et al., 2006; Pirk et al., 2011).

To the best of our knowledge, only a few low-powered studies have investigated the presence and frequency of EGFR gene mutations in MPM (Cortese et al., 2006; Enomoto et al., 2012), and none has searched for mutations in the KRAS, BRAF, and PI3KCA downstream effectors. We searched a large series of histological MPM samples for mutations in EGFR gene and its main downstream signalling effectors to evaluate their frequency and possible prognostic significance, and their possible use as predictors of the response of MPMs to targeted therapies.

**MATERIALS AND METHODS**

**Patients and samples.** The study involved 77 consecutive MPM patients admitted to the Thoracic Unit of the University Hospital of Novara between January 2008 and December 2010, all of whom were diagnosed as having MPM on the basis of multiple pleural biopsies taken by means of video-assisted thoracoscopy. The tumour samples were immediately fixed in formalin for 24 h, embedded in paraffin, and routinely processed for histology and immunohistochemistry, and the diagnosis of MPM was based on standard histological and immunohistochemical criteria, including positivity to calretinin, vimentin, and cytokeratins 5 and 6, and negativity to carcinoembryonic antigen, thyroid transcription factor 1, and Her Epy 4. The MPMs were classified on the basis of the WHO classification of pleural tumours (Travis et al., 2004), and clinically and pathologically staged on the basis of the TNM staging system (Sobin et al., 2009). The patients’ PS at the time of diagnosis was graded using the Eastern Cooperative Oncology Group (ECOG) scale (Oken et al., 1982), and the patients with a PS of 0–2 underwent therapeutic protocols indicated by the referring oncologist.

Haematoxylin/eosin-stained slides of the pleural biopsies and corresponding formalin-fixed, paraffin-embedded blocks were reviewed by a pathologist (RB) to select the area with >50% of tumour cells.

**DNA extraction.** The tumoral areas of the formalin-fixed, paraffin-embedded sections were macrodissected manually, and then five 5-μm-thick sections were prepared and collected in a 1.5 ml tube. Genomic DNA was extracted using EDTA-SDS/proteinase K followed by phenol–chloroform, and resuspended with 30 μl of DEPC-treated and RNAse-free water (Promega, Madison, WI, USA).

**Mutational analysis**

**Epidermal growth factor receptor gene.** All of the samples were analysed using the TheraScreen EGFR92 Mutation Kit (Qiagen, Manchester, UK), which combines the two technologies of ARMS and Scorpion chemistry to detect mutations in a real-time polymerase chain reaction (PCR). This kit allows the detection of in-frame deletions on exon 19, insertions on exon 20, and G719X, S768I, T790M, L858R, and L861Q mutations against a background of WT genomic DNA with a sensitivity of 1%. Starting from 2 μl of genomic DNA, the analyses were made in accordance with the manufacturer’s instruction using RotorGene Q (Qiagen), and the results were interpreted following the datasheet. To determine the presence of other less common mutations, the samples underwent further PCRs to amplify the whole sequence of exons 18–21 of the EGFR gene as described previously by Paez et al. (2004). Table 1 shows the primers and PCR conditions.

**KRAS gene.** The KRAS gene was analysed by means of a mutant-enriched PCR (ME-PCR) to detect the hotspots in codons 12 and 13 of exon 2 that include more than 95% of the known gene mutations. The ME-PCR consisted of two amplification steps (seminested PCR) in which artificial restriction sites were introduced into the wild-type amplicon using mismatched primers (Table 1). The restriction sites (BstNI for codon 12 and BglII for codon 13) introduced during the first PCR step were localised immediately next to the KRAS codon in the analysis to distinguish wild-type and mutant sequences. The wild-type amplicons were then digested by BstNI or BglII restriction enzymes, and the mutant products were enriched for a second round of amplification. The ME-PCR and digestion conditions have been described previously (Molinari et al., 2011). Mutant-enriched PCR has a sensitivity of up to 0.01%. All of the samples underwent automated sequencing by using an ABI PRISM 3130 (Applied Biosystems, Foster City; CA, USA) and reverse primers.

**BRAF gene.** Exon 15 of the BRAF gene (which contains the hotspot codon 600, where more than 90% of gene mutations occur) was analysed by means of direct sequencing in accordance with previously published protocols (Di Nicolantonio et al., 2008),
starting from 50 ng of genomic DNA. The primers and PCR conditions are shown in Table 1.

PI3KCA gene. The analysis of the PI3KCA gene was concentrated on exons 9 and 20, which include all of the hotspot codons, using previously published protocols (Sartore-Bianchi et al., 2009). The primers and PCR conditions are shown in Table 1.

Sequence analysis. All of the PCR products and KRAS second enzymatic digestions were analysed by means of 3% agarose gel electrophoresis, and then purified using NucleoSpin Gel and the enzymatic digestions were analysed by means of 3% agarose gel electrophoresis, and then purified using NucleoSpin Gel and the

Statistical analysis. This examined the correlations between the presence of gene mutations and other demographic, clinical and pathological variables. The associations between categorical variables were determined using the $\chi^2$ or Fisher’s exact test. The statistical differences of the average values were tested using a Student’s $t$-test and analysis of variance, followed by Bonferroni’s test.

The impact of the different variables on long-term outcomes was analysed using the Kaplan–Meier method of analysing disease-specific survival (DSS); the survival data were compared using the log-rank test.

P-values of $<0.05$, with a 95% confidence interval, were considered statistically significant.

RESULTS

In all, 57 patients were male (74%) and 20 were female (26%); their average age at the time of diagnosis was 68 years (range 43–90, median 64.5 years). Of these, 50 patients (64.9%) had previously been exposed to asbestos at work. Histological examination showed that 59 MPMs (77%) were epithelioid, 10 (13%) biphasic, and 8 (10.4%) sarcomatoid. In total, 41 patients had stage II tumours, 30 stage III tumours, and 6 stage IV tumours. Eastern Cooperative Oncology Group PS was 0–2 in 68 patients, and 4 or because they refused.

Follow-up data were collected from 74 patients (three were lost to follow-up). In all, 15 patients were still alive at June 2012 with a median follow-up of 24.5 months (range 14–39 months). The median DSS of the cohort as a whole was 12.5 months (range 1–39 months).

Mutation analysis. Mutations in the EGFR downstream pathway were identified in nine patients (12%): five in the KRAS gene, three in the BRAF gene, and one in the PI3KCA gene. No mutations were detected in the EGFR gene by direct sequencing or the Scorpions-ARMS assay, even though the latter has a sensitivity of 1% (vs the 10–20% of direct sequencing).

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### Table 1. Sequences of primers and PCR reaction protocol

| Gene          | Primer name | Sequence                                | Cycle                                      | Length |
|---------------|-------------|-----------------------------------------|--------------------------------------------|--------|
| KRAS codon 12 | 3F          | 5'-ACTGAAATATACCTTGTTGATGTTGACCT-3'      | 95 °C x 10 min; (95 °C x 30 s, 50 °C x 1 min, 72 °C x 1 min) x 20 cycles; 72 °C x 3 min | 143    |
|               | 10B         | 5'-ACTCATGAAATAGGTGACAGAACCCTTAT-3'     |                                            |        |
| KRAS codon 13 | 9F          | 5'-ACTGAAATATACCTTGTTGATGTTGACCT-3'      | 95 °C x 10 min; (95 °C x 30 s, 54 °C x 1 min, 72 °C x 1 min) x 20 cycles; 72 °C x 3 min | 113    |
|               | 10B         | 5'-ACTCATGAAATAGGTGACAGAACCCTTAT-3'     |                                            |        |
| KRAS codon 12 | 3F          | 5'-ACTGAAATATACCTTGTTGATGTTGACCT-3'      | 95 °C x 10 min; (95 °C x 30 s, 54 °C x 1 min, 72 °C x 1 min) x 20 cycles; 72 °C x 3 min | 143    |
|               | 14B         | 5'-TCAAAAGATAGGTGACAGAACCCTTAT-3'       |                                            |        |
| KRAS codon 13 | 9F          | 5'-ACTGAAATATACCTTGTTGATGTTGACCT-3'      | 95 °C x 10 min; (95 °C x 30 s, 54 °C x 1 min, 72 °C x 1 min) x 45 cycles; 72 °C x 3 min | 113    |
|               | 4B          | 5'-TCAAAAGATAGGTGACAGAACCCTTAT-3'       |                                            |        |
| EGFR exon 18  | EGFR18F     | 5'-TCCAGCATGCTGAGGCTGAG-3'              |                                            | 242    |
|               | EGFR18R     | 5'-GGCTCCCCACACAGACCATG-3'              |                                            |        |
| EGFR exon 19  | EGFR19F     | 5'-TGAGTGGGATGACGACGACAC-3'             | 50 °C x 2 min; 95 °C x 10 min; (95 °C x 40 s, 58 °C x 40 s, 72 °C x 35 s) x 1 min, 72 °C x 3 min | 217    |
|               | EGFR19R     | 5'-AGGTTGGGATGACGACGACAC-3'             |                                            |        |
| EGFR exon 20  | EGFR20F     | 5'-CCTCTCTCCTGACCCACATGGC-3'            |                                            | 296    |
|               | EGFR20R     | 5'-CCTCTCTCCTGACCCACATGGC-3'            |                                            |        |
| EGFR exon 21  | EGFR21F     | 5'-CCTCACGAGGCTTTCCTTC-3'               |                                            | 229    |
|               | EGFR21R     | 5'-CCTCACGAGGCTTTCCTTC-3'               |                                            |        |
| BRAF exon 15  | BRAF15F     | 5'-TCTATACGCTCCTGATAGAGG-3'             | 95 °C x 10 min; (95 °C x 15 s, 52 °C x 30 s) x 45 cycles; 72 °C x 3 min | 224    |
|               | BRAF15R     | 5'-GGCCAAATATTTATACGTGGA-3'             |                                            |        |
| PIK3CA exon 9 | PIK3CA9F    | 5'-GGGAAAAATATGGAACAAAGAAGAC-3'         | 95 °C x 10 min; (95 °C x 35 s, 56 °C x 30 s, 72 °C x 30 s) x 40 cycles; 72 °C x 10 min | 251    |
|               | PIK3CA9R    | 5'-CTGAGATCAGCCAAATCCAGTT-3'            |                                            |        |
| PIK3CA exon 20| PIK3CA20F   | 5'-CTCAATGATGCTTTCGCTTG-3'              |                                            | 241    |
|               | PIK3CA20R   | 5'-CTCAATGATGCTTTCGCTTG-3'              |                                            |        |
**KRAS and BRAF gene mutational profiling.** The KRAS gene was successfully amplified in all of the samples, five of which showed mutations: two patients had the GGT→GtT point mutation in codon 12 leading to a glycine-to-valine amino-acid substitution (G12V); two had the GGC→GaC point mutation in codon 13 leading to a glycine-to-aspartic acid substitution (G13D); and one had the rare GGC mutation in codon 13 leading to a glycine-to-aspartic acid substitution (G13S). As shown in Table 2, three of the five mutations occurred in patients with epithelioid MPMs (G12V, G13D, and G13S), one in a patient with a biphasic MPM (G13D), and one in a patient with a sarcomatoid subtype (G12V). All five patients with KRAS mutations reported previous occupational asbestos exposure.

The BRAF gene mutational analysis showed the classical valine-to-glutamic acid substitution in codon 600 (V600E) in three patients: two with epithelioid MPMs and one with a biphasic tumour (Table 2). None of them reported previous occupational asbestos exposure.

**PI3KCA gene mutational profiling.** The DNA of exons 9 and 20 of the PI3KCA gene was successfully amplified from 75 of the 77 specimens. A point mutation was detected in only one case: it occurred in exon 20, and led to a methionine-to-isoleucine substitution in position 1040 (M1040I). The patient had a biphasic mesothelioma and no previous occupational asbestos exposure (Table 2).

**Statistical analysis.** The correlations between the presence/absence of gene mutations and demographic, clinical, and pathologic features (gender, age, occupational asbestos exposure, history of previous cancer, histological type, ECOG PS, treatment) were investigated, without finding any significant differences (Table 3).

The Kaplan–Meier analysis of the influence of some variables on long-term outcomes revealed no difference in DSS between the patients with and without gene mutations \((P = 0.552)\). Moreover, separate evaluation of the patients with KRAS and BRAF mutations did not indicate any advantage in terms of DSS \((P = 0.363\) and 0.752) and, within the mutated group, no mutation significantly correlated with DSS \((KRAS, P = 0.363; BRAF, P = 0.187)\).

Interestingly, the patients with KRAS gene mutations reported occupational asbestos exposure, whereas those with BRAF and PI3KCA gene mutations did not. When the DSS of the patients with reported asbestos exposure was considered, the five KRAS gene-mutated patients had a worse prognosis than those with wild-type KRAS \((n = 42)\), although the difference was not statistically significant (mean survival, 9.20 ± 6.91 vs 15.6 ± 10.39 months; \(P = 0.188)\) (Figure 1A). On the contrary, the DSS of the patients without reported occupational asbestos exposure was better in the BRAF gene-mutated patients \((n = 3)\) than in those without BRAF mutations \((n = 22)\), although, once again, the difference was not statistically significant (mean survival, 20.33 ± 12.06 vs 12.1 ± 8.37 months; \(P = 0.140)\) (Figure 1B).

**DISCUSSION**

As EGFR is involved in the carcinogenesis of MPM, it is possible that EGFR-targeted therapies may be efficacious in MPM patients (Barbieri et al, 2011). Epidermal growth factor receptor TKI inhibitors, such as gefitinib and erlotinib, inhibit MPM cell migration and proliferation, enhance the response to radiation of human MPM cell lines, and reduce motility and invasion in MPM.

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**Table 2. Characteristics of patients with gene mutations**

| Patient | Gene | Amino-acid substitution | Gender | Age | Histotype | Asbestos exposure | DSS |
|---------|------|-------------------------|--------|-----|-----------|-------------------|-----|
| 1       | **KRAS** | G12V       | Male   | 81  | Sarcomatoid | Yes               | 4   |
| 2       | **KRAS** | G12V       | Male   | 55  | Epithelioid | Yes               | 14  |
| 3       | **KRAS** | G13D      | Male   | 82  | Epithelioid | Yes               | 4   |
| 4       | **KRAS** | G13S      | Male   | 60  | Epithelioid | Yes               | 5   |
| 5       | **KRAS** | G13D      | Male   | 77  | Biphasic  | Yes               | 19  |
| 6       | **BRAF** | V600E     | Female | 51  | Epithelioid | None              | 9   |
| 7       | **BRAF** | V600E     | Male   | 57  | Biphasic  | None              | 19  |
| 8       | **BRAF** | V600E     | Male   | 73  | Epithelioid | None              | 33  |
| 9       | **PI3KCA** | M1040I | Male   | 68  | Biphasic  | None              | 7   |

Abbreviation: DSS = disease-specific survival.

**Table 3. Statistical correlation between demographic, clinical, pathological data, and gene mutations**

|                      | Wild type \((n = 68)\) | Mutations \((n = 9)\) | \(P\)-value |
|----------------------|------------------------|----------------------|-------------|
| Age, mean ± s.d. (years) | 66 ± 21                | 67 ± 12              | 0.89        |
| Gender (male proportion) | 49/68                  | 8/9                  | 0.491       |
| Previous cancer       | 5/68                   | 2/9                  | 0.385       |
| Asbestos exposure     | 44/68                  | 6/9                  | 0.799       |
| **Histological subtype** |                       |                      |             |
| Epithelial           | 54/68                  | 5/9                  | 0.241       |
| Biphasic             | 7/68                   | 3/9                  | 0.135       |
| Sarcomatoid          | 7/68                   | 1/9                  | 0.623       |
| **ECOG score**       |                        |                      |             |
| 0–2                  | 60/68                  | 8/9                  | 0.892       |
| >2                   | 8/68                   | 1/9                  | 0.617       |
| **Clinical stage**   |                        |                      |             |
| II                   | 37/68                  | 4/9                  | 0.689       |
| III–IV               | 31/68                  | 5/9                  | 0.576       |
| **Treatment type**   |                        |                      |             |
| None                 | 13/68                  | 1/9                  | 0.623       |
| Platinum             | 19/68                  | 3/9                  | 0.876       |
| Platinum + pemetrexed | 36/68                  | 5/9                  | 0.776       |

Abbreviations: ECOG – Eastern Cooperative Oncology Group; s.d. – standard deviation.
cell lines (Kurai et al., 2012). However, the promising results obtained in in vitro studies were not reproduced in two phase II trials involving patients with pleural and peritoneal mesotheliomas, although it should be noted that neither study evaluated the in vivo effects of anti-EGFR mAbs on MPMs, although one recent study has found that cetuximab is highly efficacious in cultured MPM cell lines (Kurai et al., 2012). It has been demonstrated that mutations in EGFR downstream pathways may affect the efficacy of EGFR mAbs in other tumours such as colorectal adenocarcinoma (Jonker et al., 2007), and we found nine patients (11.7%) with missense mutations involving the KRAS gene-mutated patients (9.20 ± 3.69 months) and the Braf gene-mutated patients (10.39 ± 3.57 months). Furthermore, some of the detected mutations have never been reported before, and their biological and clinical significance is still unknown.

An alternative method of blocking EGFR is to use mAbs, which may be extremely useful as it has been demonstrated that MPM patients show EGFR gene amplification (Dazzi et al., 1990; Destro et al., 2006; Okuda et al., 2008). No studies have been published which provide evidence that cetuximab is efficacious in MPM patients. However, the study involved Japanese patients, who are characterised by more Western patients (Endo et al., 2005). Hence, it is possible that the percentage of KRAS gene-mutated cells is very low in MPM and that more widely used sequencing methods are unable to detect small clones.

Our findings show that, although infrequent, mutations in EGFR downstream pathways may be found in MPMs, thus supporting the hypothesis that EGFR mAbs may be clinically effective in the majority of patients. On the other hand, patients with a molecular profile indicating putative resistance to EGFR mAbs (because of the presence of KRAS or Braf or PIK3CA mutations) may be directed towards new targeted therapies. One recent study has shown that vemurafenib is promising not only in patients with metastatic melanoma but also in patients with non-small-cell lung cancer carrying a Braf mutation (Gautschi et al., 2012), and selumetinib and BYL-719, which target KRAS and PIK3CA mutations, are currently being evaluated in several clinical trials (http://ctcmagnifier.org/). Our data therefore underline the importance of the molecular characterisation of patients with MPM.

The clinical implications of the gene mutations detected in our study are not clear. DSS was no different in the patients with or without gene mutations (whether analysed together or separately). Interestingly, all of the patients with KRAS gene mutations reported occupational asbestos exposure, but none of those with Braf or PIK3CA gene mutations. Comparison of mean DSS in the KRAS and Braf gene-mutated patients vs wild-type patients previously exposed to asbestos or not, showed that the KRAS gene-mutated patients (n = 5) tended to have a worse prognosis than the wild-type patients (9.20 ± 6.91 vs 15.6 ± 10.39 months), and the Braf gene-mutated patients (n = 3) tended to have a better
prognosis (20.33 ± 12.06 vs 12.1 ± 8.37 months). However, the differences were not statistically significant and our findings need to be confirmed in larger series of MPM patients.

In conclusion, our extensive molecular characterisation of EGFR pathways may explain the failure of TKI administration and may open up the possibility of developing new targeted therapies.

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