

Patients with peritoneal mesothelioma lack epidermal growth factor receptor tyrosine kinase mutations that would make them sensitive to tyrosine kinase inhibitors

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Abstract. The epidermal growth factor receptor (EGFR) is a promising target for cancer therapy. The presence of certain somatic mutations in the tyrosine kinase (TK) domain of the *EGFR* gene is associated with clinical response to TK inhibitors (TKI) in patients with lung adenocarcinoma. In this study we evaluated the status of somatic mutations in the entire TK domain of the *EGFR* gene by direct sequencing using early passage peritoneal mesothelioma cells, established cell lines as well as 33 peritoneal mesothelioma tumor samples. No novel mutations were found in the cell lines. Sequence analysis of the EGFR TK domain revealed the presence of a silent polymorphism (c.2607G→A, Q787Q) at exon 20 of both peritoneal mesothelioma cell lines as well as tumor specimens. The frequency of genotypes AA and GA was 42.8 and 57.2% in the cell lines and 33.3 and 57.6% in tumor specimens, respectively. The TKI erlotinib showed an IC_{50} in the range of 10–50 μ M in five out of the seven cell lines with a GA genotype while all five cell lines with the AA genotype had an IC_{50} >50 μ M. Of the 33 peritoneal mesothelioma tumor samples analyzed none had an EGFR TKI sensitizing mutation and only one specimen showed an earlier reported somatic mutation at codon 850 in exon 21 of the *EGFR* gene. Our data show that patients with peritoneal mesothelioma do not harbor somatic mutations in the EGFR TK domain that would make them sensitive to EGFR TKI.

Introduction

Malignant mesothelioma is a tumor of the lining of the pleural and peritoneal cavities that is associated with prior asbestos exposure (1). However, 15–20% of cases with mesothelioma have no history of exposure to asbestos. An estimated 3,000 new cases per year are diagnosed in the United States out of which diffuse peritoneal mesothelioma accounts for approximately 10–30% of the cases (2). In patients with pleural mesothelioma aggressive surgical resection is an option in some patients with early stage disease. In a large randomized phase III clinical trial of patients with unresectable disease, systemic chemotherapy with pemetrexed and cisplatin resulted in improved overall survival as compared to cisplatin alone and is the current standard of care. However, the median survival of these patients was only 12.1 months (3). In contrast to patients with pleural mesothelioma, the prognosis of patients with peritoneal mesothelioma is significantly better, with a median overall survival greater than 5 years in those patients who are candidates for cytoreductive surgery and intraoperative chemotherapy (4,5).

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases (TK) and it plays a major role in proliferation, invasion, metastasis and survival of cancer cells (6). EGFR is expressed in a variety of human epithelial tumors including lung, head and neck, colorectal and breast cancers (7). EGFR is also overexpressed in 40–60% of the malignant mesothelioma cases (8,9). However, EGFR protein expression alone does not correlate with the clinical response to anti-EGFR therapy and thus does not show the dependence of the tumor on the EGFR pathway (8). Some patients with lung cancer have mutations in the EGFR that make them very sensitive to treatment with EGFR TK inhibitors (TKI), such as erlotinib and gefitinib (10,11). The presence of activating mutations (especially deletion in exon 19 and missense mutation in exon 21) in the TK domain enhances the kinase activity and maintains the EGFR in a constitutively active form with tumor growth dependent on EGFR signaling (12). Success in treatment with TKI in lung cancer patients has prompted the identification of these activating mutations in other tumors, including malignant mesothelioma. However,

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Abbreviations: EGFR, epidermal growth factor receptor; TK, tyrosine kinase; TKI, TK inhibitor

Key words: EGFR, peritoneal mesothelioma, somatic mutation, tyrosine kinase inhibitor, erlotinib

Table I. Primers and PCR conditions for 7 exons of the EGFR TK domain.

Exons	Primer sequence 5'→3'	Annealing temperature (°C)	PCR product size (bp)
Exon 18			
Forward	GCT GAG GTG ACC CTT GTC TC	58	250
Reverse	CCA CAG CTT GCA AGG ACT CTG G		
Exon 19			
Forward	ACA ATT GCC AGT TAA CGT CTT CC	58	199
Reverse	CCA AAA GGT GGG CCT GAG GTT CA		
Exon 20			
Forward	CCA CCA TGC GAA GCC ACA CT	58	261
Reverse	CCT CCC CTC CCC GTA TCT CCC T		
Exon 21			
Forward	TGA TCT GTC CCT CAC AGC AG	58	226
Reverse	CCA ATG CTG GCT GAC CTA AAG C		
Exon 22			
Forward	TCC AGA GTG AGT TAA CTT TTT CCA	56	277
Reverse	TTG CAT GTC AGA GGA TAT AAT GTA A		
Exon 23			
Forward	CTA AAG CAA GGG ATT GTG ATT G	58	259
Reverse	CCA GGC TCA GCT AGG CAG TGT GGA		
Exon 24			
Forward	GCA ATG CCA TCT TTA TCA TTT C	56	281
Reverse	GCT GGC ATG TGA CAG AAC AC		

mutation in the TK domain of the *EGFR* gene are not present in malignant pleural mesothelioma (8,13,14). Furthermore, phase II clinical trials of TKI gefitinib (15) and erlotinib (16) in malignant pleural mesothelioma patients did not demonstrate any activity.

However, Foster *et al* described the presence of somatic EGFR TK mutations in 9 of 29 (31%) patients with peritoneal mesothelioma (17). They screened the whole TK domain (exons 18-24) using denaturing high performance liquid chromatography and confirmed the presence of mutation by sequencing. They found the presence of 7 novel mutations and one already known sensitizing mutation (L858R). All of these novel mutations were shown to activate the TK activity in a transfected cell line and were sensitive to the EGFR inhibitor erlotinib *in vitro* (18). Given the potential clinical implications of these findings, we conducted this study to evaluate EGFR mutations/polymorphisms in the TK domain of early passage peritoneal mesothelioma cell cultures, in established malignant peritoneal mesothelioma cell lines and in peritoneal mesothelioma tumor samples by completing direct sequencing. We also wished to correlate the effect of EGFR TKI erlotinib on cell proliferation and its correlation with any potential mutation/polymorphism encountered.

Materials and methods

Reagents and cell culture. Erlotinib was procured from Active Biochem (Maplewood, NJ). Cell culture related reagents were

purchased from Invitrogen/Life Technologies, Inc. (Rockville, MD). FBS was purchased from Lonza Walkersville, Inc. (Walkersville, MD). Cells were cultured in RPMI-1640 supplemented with 10% FBS, 2 mM glutamine and 10 µg/ml penicillin/streptomycin at 37°C in 5% CO₂ humidified air.

Patient samples. Archival tumor samples were obtained from patients with peritoneal mesothelioma treated on clinical protocols approved by the National Cancer Institute (NCI) Institutional Review Board. Early passage peritoneal mesothelioma cell cultures were established from the ascites of patients with peritoneal mesothelioma treated on NCI IRB approved protocols. In addition, established peritoneal mesothelioma cell lines were also evaluated for EGFR TK mutations.

Pathological examination of tumor samples. The patients whose tumor samples were used to determine somatic EGFR mutations were obtained from the archival files of the NCI. The diagnosis of peritoneal mesothelioma was confirmed by the pathologist (M.R.). Unstained slides were cut from the tumor blocks that showed mesothelioma. These unstained slides were then evaluated by the pathologist to identify the tumor area for DNA extraction.

Polymerase chain reaction and direct sequencing of EGFR. Genomic DNA was isolated from cell lines, as well as from tumors embedded in paraffin blocks using ultra clean tissue and cells with the DNA isolation kit from Mo Bio Laboratories

Table II. Presence of EGFR mutation/polymorphisms in malignant peritoneal mesothelioma cells and sensitivity to erlotinib.

Peritoneal mesothelioma cells	c.2062-104C→A (Intronic change)	c.2284-60T→C (Intronic change)	Q787Q (c.2607 G→A) (Exon 20)	T903T (c.2709 T→C) (Exon 23)	Sensitivity of peritoneal mesothelioma cells to erlotinib (IC ₅₀)
Early passage cells (n=8)					
NCI-M-03	Wt	Wt	Hz	Wt	ND
NCI-M-05	Wt	Wt	Hz	Hz	>50 μM
NCI-M-07	Hz	Wt	Hm	Wt	>100 μM
NCI-M-09	Wt	Wt	Hz	Hz	10 μM
NCI-M-10	Wt	Hz	Hz	Wt	20 μM
NCI-M-11	Hz	Hz	Hm	Wt	ND
NCI-M-14	Wt	Wt	Hz	Wt	>50 μM
NCI-M-17	Wt	Hz	Hm	Wt	>50 μM
Established cell lines (n=6)					
ORT	Wt	Wt	Hz	Wt	10 μM
ROB	Wt	Wt	Hz	Wt	10 μM
YOU	Wt	Hz	Hz	Wt	40 μM
PET	Wt	Hz	Hm	Wt	>50 μM
HAY	Wt	Hz	Hm	Wt	>100 μM
HEC	Wt	Hz	Hm	Wt	>50 μM

ND, not done; Wt, wild-type; Hz, heterozygous; Hm, homozygous.

(Carlsbad, CA) and the QIAamp DNA FFPE tissue kit from Qiagen (Valencia, CA), respectively. Briefly, DNA was amplified in a total volume of 25 μl, containing 1.5 mM MgCl₂, 200 μM of each dNTP (dATP, dCTP, dGTP and dTTP), 10 pM of each primer, 0.3 units Taq DNA polymerase, and 100 ng of DNA in 1X Taq polymerase buffer. The primers used to amplify exons 18-24 of the EGFR gene are listed in Table I. DNA was first denatured at 95°C for 5 min, followed by 35 cycles of PCR with denaturation at 94°C for 45 sec, primer annealing for 45 sec at 56°C or 58°C and primer extension for 45 sec at 72°C. At the end of the last cycle, the mixture was incubated at 72°C for 5 min. The sequencing reaction was performed in a final volume of 20 μl containing 20 pmol of one primer (forward or reverse), 3 μl of BigDye (version 1.1) and 2 μl of purified PCR product. Twenty-five cycles were performed: denaturation at 96°C for 10 sec, primer annealing at 55°C for 10 sec and extension at 60°C for 4 min. Direct sequencing of amplified PCR products was performed on an ABI Prism sequencing apparatus (ABI Prism 310 Genetic Analyzer, Applied Biosystems, Foster City, CA).

Growth inhibition of early passage mesothelioma cell cultures and established cell lines. Cells were seeded in 96-well plates at 1x10⁴ cells/well in 200 μl of medium. After seeding for 24 h, cells were treated with various concentrations (0.1-100 μM) of erlotinib for 72 h. The inhibition of cell growth was assessed using the WST assay from Dojindo Molecular Technologies (Rockville, MD). Relative growth inhibition rates for the vehicle control (DMSO) were calculated and IC₅₀ values were determined as the drug concentrations associated with 50% survival of cells.

Results

Absence of EGFR mutations in early passage and in established cell lines. There were no mutations present in the TK domain of peritoneal mesothelioma cell lines. However, we found the commonly known single nucleotide polymorphism (SNP) c.2607G→A (rs1050171, Q787Q glutamine, CAG to CAA) in exon 20 of all cell lines either in the heterozygous (GA, 57.2%) or the homozygous form (AA, 42.8%) (Table II and Fig. 1). None of the cell lines had the GG genotype. The other SNPs that were found in these cells included earlier reported c.2709T→C (rs1140475, T903T, threonine, ACT to ACC) in exon 23, c.2284-60T→C (rs10241451) and c.2062-104C→A (rs17337100) in the intronic region of the EGFR gene (Fig. 1). The c.2062-104C→A and c.2709T→C were present in 13.3% and the c.2284-60T→C in 46.6% of the peritoneal mesothelioma cell lines, respectively, in the heterozygous form. To our knowledge this is the first report identifying the presence of these SNPs in mesothelioma since earlier studies only looked for the presence of specific mutations which are commonly found in lung cancer.

Although the significance of these SNPs is unknown, we wanted to examine whether these SNPs might play some role in the sensitivity of these cell lines to the TKI erlotinib. We found that peritoneal mesothelioma cell lines having homozygous AA genotype for c.2607G→A were completely resistant (IC₅₀ >50 μM) to erlotinib while 5 out of the 7 cell lines with heterozygous GA genotype showed moderate sensitivity (IC₅₀ <50 μM) to treatment with erlotinib although this is not in the clinically relevant dose range (Table II). No correlation could be seen with the presence of other SNPs and the sensitivity of

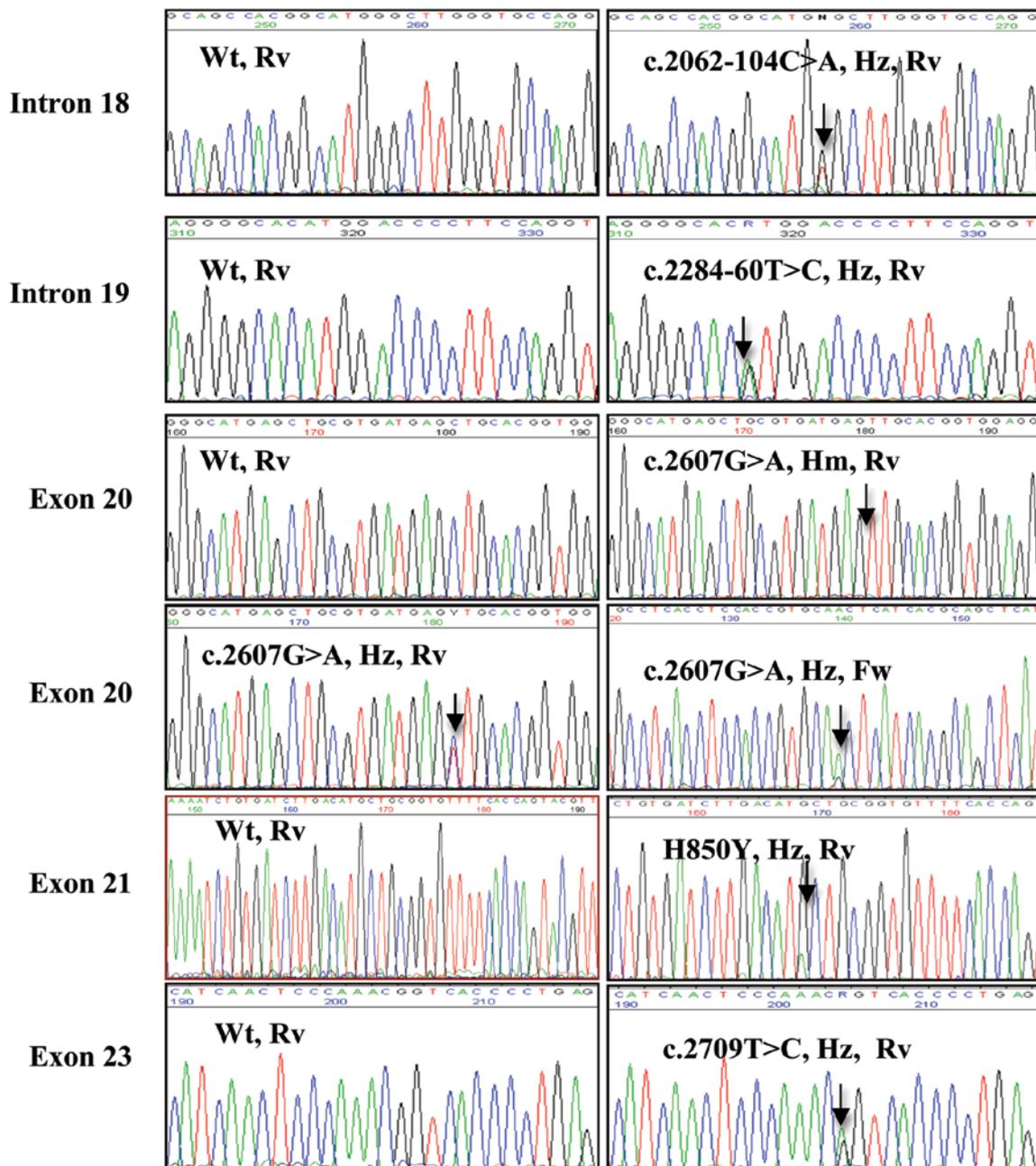


Figure 1. Identification of mutation/polymorphisms in malignant mesothelioma. Respective chromatogram of each mutation/polymorphism has been shown. Change of position is indicated by an arrow. Wt, wild-type; Hz, heterozygous; Hm, homozygous; Fw, forward primer; Rv, reverse primer.

these cell lines towards erlotinib. There was also no correlation between the expression of EGFR protein in these cell lines and their sensitivity to erlotinib (data not shown). Our results show that none of the cell lines is highly sensitive to erlotinib indicating that absence of sensitizing mutations in the *EGFR* gene contributes towards resistance to TKI therapy.

Absence of somatic EGFR mutations in peritoneal mesothelioma tumor samples. Out of the 33 cases analyzed, 23 were male and 10 were female ranging from 19-75 years of age (Table III). All patients had epithelial histology with mostly high-grade tumors. Out of these 33 patients, 25 underwent cytoreductive surgery with intraperitoneal hyperthermic chemotherapy while 8 patients had exploratory laparotomy.

DNA was extracted from these 33 formalin-fixed paraffin-embedded tumor samples and the quality of the DNA was checked using PCR amplification of β -actin as a quality control gene (data not shown). Of the 33 specimens we found only one case with a single mutation in exon 21 at codon 850 (H850Y, CAT to TAT, c.2548C→T) of the *EGFR* gene (Table III and Fig. 1). No other previously reported or novel *EGFR* mutations were found. However, we found the same pattern of SNPs that were present in peritoneal mesothelioma cell lines. The SNP c.2607G→A was present in 91% of malignant mesothelioma cases. Eleven of the 33 samples had the AA genotype (33.3%) and 19 had the AG genotype (57.6%) while 3 patients (9.1%) had the prototype GG genotype (Table III). The TC genotype of c.2709 T→C was present in 9/33 cases (27.3%) and the CC

Table III. Presence of EGFR mutation/polymorphisms in malignant peritoneal mesothelioma tumor samples.

Patient	Gender	Age (years)	c.2062-104C→A (Intronic change)	c.2284-60T→C (Intronic change)	Q787Q (c.2607 G→A) (Exon 20)	H850Y (c.2548 C→T) (Exon 21)	T903T (c.2709 T→C) (Exon 23)
1	M	65	Wt	Wt	Hm	Wt	Wt
2	F	71	Wt	Hz	Hz	Wt	Wt
3	F	51	Wt	Hz	Hm	Wt	Wt
4	M	41	Wt	Wt	Hz	Wt	Hz
5	M	58	Wt	Wt	Hz	H850Y	Hz
6	M	53	Hz	Wt	Hz	Wt	Wt
7	F	19	Wt	Hz	Hz	Wt	Hz
8	M	35	Wt	Wt	Hz	Wt	Wt
9	M	78	Wt	Hz	Hm	Wt	Wt
10	M	31	Wt	Wt	Hz	Wt	Wt
11	M	51	Wt	Wt	Hz	Wt	Wt
12	M	50	Wt	Wt	Hz	Wt	Hz
13	M	57	Wt	Wt	Wt	Wt	Wt
14	F	54	Wt	Wt	Hm	Wt	Hm
15	M	75	Wt	Wt	Wt	Wt	Wt
16	M	42	Wt	Hz	Hm	Wt	Wt
17	F	63	Wt	Wt	Hm	Wt	Hz
18	F	65	Wt	Wt	Wt	Wt	Hz
19	M	60	Wt	Wt	Hz	Wt	Wt
20	F	72	Wt	Wt	Hz	Wt	Hz
21	M	69	Wt	Wt	Hm	Wt	Wt
22	M	29	Wt	Hz	Hm	Wt	Wt
23	M	54	Wt	Wt	Hm	Wt	Wt
24	M	54	Wt	Wt	Hm	Wt	Wt
25	M	38	Wt	Wt	Hz	Wt	Wt
26	M	57	Wt	Wt	Hz	Wt	Wt
27	F	54	Wt	Wt	Hm	Wt	Wt
28	M	63	Wt	Wt	Hz	Wt	Wt
29	M	68	Wt	Wt	Hm	Wt	Wt
30	F	57	Wt	Wt	Hm	Wt	Wt
31	M	56	Wt	Wt	Hz	Wt	Hz
32	F	75	Wt	Wt	Hz	Wt	Wt
33	M	59	Wt	Wt	Hz	Wt	Hz

M, male; F, female; Wt, wild-type; Hz, heterozygous; Hm, homozygous.

genotype in 1/33 cases (3.03%). The intronic polymorphisms c.2062-104C→A and c.2284-60T→C were present in 1/33 (3.03%) and 6/33 (19%) of cases, respectively, in the heterozygous form (Table III).

Discussion

EGFR somatic mutations are present in some patients with lung cancer and are associated with sensitivity to EGFR TKI. However, no such mutations are found in patients with pleural mesothelioma and clinical trials of EGFR TKI have no activity in this disease. However, a recent report suggested

that EGFR somatic mutations are frequent in peritoneal mesothelioma patients (17). Since these findings could have clinical implications we conducted a comprehensive analysis of EGFR TK mutation analysis by direct sequencing of the entire TK domain of the *EGFR* gene using early passage peritoneal mesothelioma cell cultures, established peritoneal mesothelioma cell lines and tumor samples from patients with peritoneal mesothelioma.

In both the mesothelioma cells and tumor specimens we observed several SNPs. The most commonly found polymorphism in peritoneal mesothelioma cases was c.2607G→A, present in exon 20 of the *EGFR* gene. This polymorphism has

been reported earlier in a wide variety of tumors including gastric (19), lung (20), cervical (21), pancreatic (22) and head and neck (23). Zhang *et al* have shown the association of the AA genotype of c.2607G→A with an increased risk of lung cancer (24), although they did not show any correlation between this SNP and somatic EGFR mutations found in those samples. In head and neck cancer cell lines the GA genotype showed higher sensitivity to gefitinib than the GG genotype (25). A study by Sasaki *et al* has shown a weak association between the SNP (GA/AA genotype) and a worse outcome with gefitinib treatment in Japanese lung cancer patients (26). All of these findings indicate that this SNP may affect either the stability of the EGFR protein, the rate of translation, or possibly gene transcription (24,26). We also found a moderate response to erlotinib *in vitro* in some of the peritoneal mesothelioma cell lines with the GA genotype while cells with the AA genotype were completely resistant to erlotinib.

Direct sequencing of the entire TK domain of the 33 tumor samples from patients with malignant peritoneal mesothelioma did not identify any novel mutations or presence of mutations that are associated with sensitivity to EGFR TKI. Only once case showed the presence of a mutation in exon 21 (H850Y) of the *EGFR* gene. Mutation at codon 850 has been reported previously in other cancers although the change in amino acid was different from what we found in our study. Jia and Chen reported the presence of H850D mutation in one patient with adenocarcinoma of the lung (20). The mutation coexisted with the silent SNP c.2607G→A. Yang *et al* showed the presence of the H850R mutation together with the EGFR sensitizing mutation L858R in two patients with lung cancer (27).

The results of our study are in contrast to the report by Foster *et al* who described the presence of somatic EGFR mutations in 9 out of 29 cases with peritoneal mesothelioma (17,18). The reason for these desperate results is not clear. However, our results are in agreement with the study by Perrone *et al* who found no EGFR mutations in 20 patients with malignant peritoneal mesothelioma (28). Perrone *et al* performed the direct sequencing only for exons 18-21 where the activating mutations of the *EGFR* gene are present, whereas we performed the DNA sequencing of the entire TK domain (exons 18-24) to exclude the presence of any novel mutation.

In conclusion, direct sequencing of the entire EGFR TK domain, using 14 mesothelioma cell lines and 33 patient samples, did not reveal any EGFR sensitizing mutations. Therefore, we conclude that EGFR TKI will not be useful for the treatment of patients with peritoneal mesothelioma.

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