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Increased expression and no mutation of the *Flap endonuclease* (*FEN1*) gene in human lung cancer

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The underlying molecular mechanisms leading to microsatellite alteration and mutations in human lung cancer remain unknown. Since *Flap endonuclease1* (*Fen1*), which functions in the base excision repair system, has been shown to be involved in tumor progression of mouse models with microsatellite instability in a haplo-insufficient manner, we performed expression and mutation analyses for *FEN1* in human lung cancer cell lines. Reverse transcriptase PCR analysis revealed that all 49 lung cancer cell lines (20 small cell lung cancers (SCLCs) and 29 non-small cell lung cancers (NSCLCs)) expressed *FEN1*. In addition, microarray analysis showed that *FEN1* expression was elevated significantly by 1.65-fold ($P = 0.001$) in SCLC cell lines compared to normal lung controls (normal human lung cultures and immortalized normal human bronchial epithelial cell lines). FEN1 protein was abundantly expressed in all 23 lung cancer cell lines (10 SCLCs and 13 NSCLCs) and was expressed at lower levels in three of four normal lung epithelial culture controls. Direct sequencing of genomic DNAs revealed no *FEN1* mutation in seven SCLCs and nine NSCLCs. As part of this analysis we discovered and sequenced a *FEN1* pseudogene (GenBank accession #AY249897) located at 1p22.2. This pseudogene is amplified from cDNA preparations contaminated with genomic DNA and must be taken into account in any *FEN1* mutation analysis studies. Our results suggest that alterations of *FEN1* are not likely to contribute to development of lung cancer.
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Human lung cancers show several distinct types of genomic abnormalities, one of which appears as chromosomal abnormalities with allele loss including but not limited to frequent deletions at 3p, 9p21, 13q14 and 17p13 (sites of known tumor suppressor genes) or amplification of dominant oncogene sites, which are observed in almost all lung cancers (Girard *et al*., 2000; Sekido *et al*., 2003; Zochbauer-Muller *et al*., 2002). Another type of abnormality appears as abnormal size microsatellite repeats at polymorphic loci termed microsatellite alterations. This latter type is seen in ~35% of small cell lung cancer (SCLC) and ~22% non-small cell lung cancer (NSCLC) (Sekido *et al*., 2003). A related phenotype known as microsatellite instability (MSI) in hereditary nonpolyposis colon cancers (HNPCCs) is a well-characterized oncogenic phenotype (Aaltonen *et al*., 1993; Ionov *et al*., 1993; Thibodeau *et al*., 1993), and represents a colorectal cancer predisposition syndrome. However, microsatellite alteration phenotype seen in lung cancer is distinct from MSI. MSI phenotype is defined as a high frequency of alterations in mono- or dinucleotide repeats and these alterations are more frequent than alterations in tri- or tetranucleotide repeats (Boland *et al*., 1998). However, alterations in microsatellite loci in lung cancer occur more frequently in tri- or tetranucleotide than in mono- or dinucleotide (Boland *et al*., 1998). In addition, although most HNPCC cases and their associated MSI are accounted for by mutation of DNA mismatch repair (MMR) genes (Thibodeau *et al*., 1996; Peltomaki and Vasen, 1997; Wijnen *et al*., 1998; Yan *et al*., 2000; Charbonnier *et al*., 2002), MMR gene mutations have not been demonstrated in lung cancer. Thus, microsatellite alterations in lung cancer are different from MSI and its underlying mechanisms remain unknown.

Recently, *the Flap endonuclease (Fen1)* gene has been demonstrated to be involved in mouse gastrointestinal tract cancer in a haplo-insufficient manner (Kucherlapati *et al*., 2002). In this study, *Fen1* heterozygous mice generated by gene knockout appeared normal. However, when combined with a mutation of adenomatous polyposis coli gene (*APC*), they showed increased number of adenocarcinomas and decreased survival. Further, the tumors from these mice showed MSI, suggesting that decreased expression of *Fen1* may be attributable to MSI of these cancer cells. FEN1 is responsible for DNA replication and base excision

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repair (BER) pathways and has been shown to play an important role in the integrity of genome (Lindahl, 1971; Guggenheimer *et al*., 1984; Siegal *et al*., 1992; Harrington and Lieber, 1994, 1995; Kim *et al*., 1998). In addition, there is an evidence that alteration of the BER system occurs in human lung cancer. DNA polymerase *β*-pol, which functions in gap-filling synthesis in BER pathway, has been shown to be abnormally expressed in some human lung cancers (Bhattacharyya *et al*., 1999). Finally, lung cancers show frequent loss of expression of *APC* secondary to tumor-acquired promoter methylation (Virmani *et al*., 2001). With this background, we hypothesized that alteration of *FEN1* might occur in lung cancer cells, and this inactivation combined with *APC* inactivation could lead to lung cancer pathogenesis by mechanisms similar to those seen in the mouse colon tumor model. To examine this hypothesis we studied expression and mutation of *FEN1* in human lung cancer cell lines.

We performed reverse transcriptase PCR (RT–PCR) analysis for *FEN1* and found that all 49 lung cancer cell lines (20 SCLCs and 29 NSCLCs) showed expression of *FEN1* (Figure 1). As part of this analysis we discovered and partially sequenced a *FEN1* pseudogene (GenBank accession #AY249897). To evaluate the mRNA expression of *FEN1* quantitatively, we next performed microarray analysis using 52 lung cancer cell lines (21 SCLCs and 31 NSCLCs) and four normal lung controls including two normal lung epithelial cultures (NHBE and SAEC) from Clonetics (San Diego, CA, USA) and two immortalized normal human bronchial epithelial cell lines (BEAS-2B and HCCBE-1) as controls (see legend of Figure 1 for details). The HCCBE-1, immortalized normal human bronchial epithelial cell line, was established by us via introducing mouse cyclin-dependent kinase 4 and human *hTERT* genes into normal human bronchial epithelial cell cultures obtained from a nonsmoking 68-year-old woman with lung metastases from endometrial cancer (Ramirez, manuscript in preparation). Normalized fluorescence intensities of *FEN1* expression in SCLCs, NSCLCs and normal controls were 754 ± 199 (mean $\pm$ s.d.), 528 ± 159 and 457 ± 94, respectively. *FEN1* expression in SCLCs was significantly elevated by 1.65-fold (nonpaired Student's *t*-test, $P = 0.001$) compared to normal controls. No cell lines lacked expression of *FEN1*.

To evaluate FEN1 expression at the protein level, we performed Western blots for FEN1. In total, 23 lung cancer cell lines (10 SCLCs and 13 NSCLCs) and four normal lung epithelial culture controls (HCCBE-1, BEAS2B, NHBE and SAEC) were analysed. While all tested lung cancer cell lines expressed FEN1 protein abundantly, except for BEAS-2B, the normal lung controls expressed FEN1 at lower levels compared to lung cancer cell lines (Figure 2).

We next performed a mutation search for *FEN1* by direct DNA sequencing. Seven SCLCs (NCI-H69, NCI-H182, NCI-H748, NCI-H1437, NCI-H1514, NCI-H1522 and NCI-H2198) and nine NSCLCs (NCI-H157, NCI-H358, NCI-H460, NCI-H1264, NCI-H1334, NCI-H1648, NCI-H1781, NCI-H1993 and NCI-H2110) cell

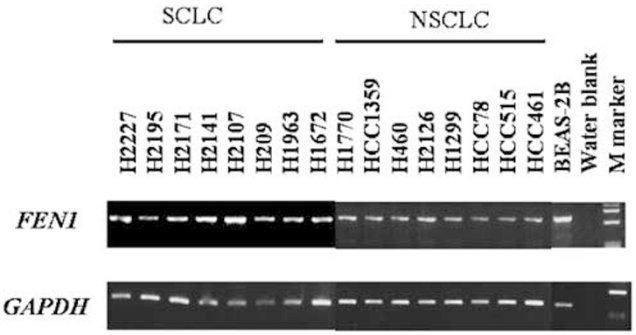

Figure 1 Representative RT–PCR analysis of *FEN1* in lung cancer cell lines. All cell lines express *FEN1* at detectable level. *GAPDH* is used as a control for the quality of cDNA. For the RT–PCR analysis 49 lung tumor cell lines (20 SCLCs and 29 NSCLCs) and BEAS-2B cell line as a control were studied. The lung tumor cell lines were from the NCI (prefix (NCI-H)) or the Hamon Cancer Center (prefix (HCC)), most of which have been described and are deposited at the American Type Culture Collection (ATCC) (Phelps *et al*., 1996; Wistuba *et al*., 1999). BEAS-2B, immortalized normal human bronchial epithelial cell lines, was kindly provided by Dr Jerry W Shay (University of Texas Southwestern Medical Center). The lung cancer cell lines studied included: 20 SCLCs (H82, H128, H146, H209, H289, H378, H524, H526, H889, H1184, H1607, H1672, H1963, H2107, H2141, H2171, H2195, H2227, HCC33 and HCC970); and 29 NSCLCs (H157, H358, H460, H1299, H1395, H1437, H1648, H1770, H1993, H2009, H2087, H2122, H2126, H2347, H2882, H2887, HCC15, HCC44, HCC78, HCC95, HCC193, HCC366, HCC461, HCC515, HCC827, HCC1171, HCC1359, HCC1833 and HCC2450). To avoid the contamination of PCR products amplified from genomic DNA, FEN1-S1381 (forward) and FEN1-AS4397 (reverse) primers were designed in exons 1 and 2 of *FEN1*, respectively. FEN1-AS4397 primer was designed to avoid obtaining a PCR product from the *FEN1* pseudogene. The following reaction and cycling conditions were used for both *FEN1*and *GAPDH*. The reaction was performed in a 25-μl mixture containing $10 \times$ PCR buffer (QIAGEN), deoxynucleotide triphosphate (1.25 mM), primers (final concentration, 1.0 μM), 1 U of HotStar Taq (QIAGEN), and cDNA (1 μl). Amplification was carried out in a 9600 Perkin-Elmer Thermal Cycler. Cycling conditions were one cycle of 95°C for 15 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. The final extension was at 72°C for 7 min. Amplified PCR products were electrophoresized on 1% agarose gel with ethidium bromide. The sequences of primers used for RT–PCR are FEN1-S1381; TTT AGC CGC CGA GGC CGC GTG TC, FEN1-AS4397; ACT GTT CCT GGT TCA GGC, *GAPDH*-forward; CAT GAC AAC TTT GGT ATC GTG, GAPDH-reverse; GTG TCG CTG TTG AAG TCA GA. The sequences of primers used for genomic PCR for direct sequencing are FEN1S3680; ATA ACC TTT CTC CTT TCC TCC GTC, FEN1AS4931; AGC TCT TAA GGG TAC AAG ACG GC. The HG-U133A chip from Affymetrix was used for microarray analysis. Extracted RNA was analysed for quality on RNA 6000 Nano kit (Agilent Technologies) with Agilent Bioanalyzer software or on regular agarose–formaldehyde gels. A measure of 5 μg of total RNA was used on a single round of amplification. The Affymetrix protocol started with cDNA synthesis, using a poly (T) primer with a T7 promoter. The double-stranded cDNA generated was then used to prime the synthesis of cRNA using biotinylated ribonucleotides (UTP and CTP). After the labeled cRNA was synthesized, it was fragmented and hybridized to the GeneChip at 45°C for 16 h in a rotary incubator. After hybridization the analyte solution was removed and the array washed and stained with Streptavidin Phycoerythrin in the Affymetrix GeneChip fluidics station. After washing, the array was scanned (Agilent GeneArray Scanner) and the data extracted with the MicroArray Suite 5.0 software (Affymetrix). Expression signals were median-normalized and the difference in the expression levels of *FEN1* between cancer cell lines and normal controls was analysed by nonpaired *t*-test

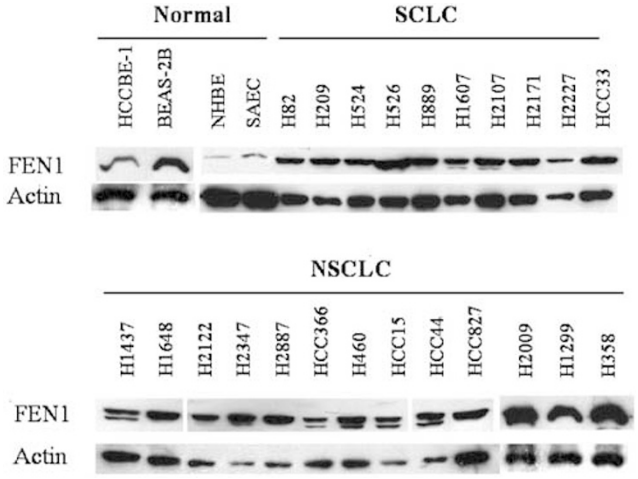

Figure 2 Western blot of FEN1 in lung cancer cell lines. All tested lung cancer cell lines expressed FEN1 protein abundantly, and except for BEAS-2B, normal lung controls expressed FEN1 at lower levels compared to lung cancer cell lines. Two immunoreactive bands were detected in some samples. The minor faster migrating band may be an unknown cross-reacting protein or proteolytic fragment of FEN1. Actin protein levels were used as a control for protein loading. Cells were grown and harvested at 80–90% confluency, and cellular proteins were extracted with lysis buffer (40 mM HEPES–NaOH, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl) containing Complete Mini, a cocktail of protease inhibitors (Roche, IN, USA). Total protein was separated on SDS–PAGE and electroblotted to nitrocellulose membranes (Schleicher & Schuell, NH, USA). After blocking with 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline, the membranes were incubated at room temperature for 1 h with the rabbit polyclonal anti-FEN1 (Santa Cruz, CA, USA), and mouse monoclonal anti-Actin (Sigma, MO, USA) antibodies. The membranes then were developed with peroxidase-labeled antibodies (Amersham Pharmacia, NJ, USA) with the Super Signal chemiluminescent substrate (Pierce, IL, USA)

lines were studied, but no mutations and no polymorphisms of *FEN1* were found in genomic DNAs from these tumor cell lines by sequencing PCR products.

In this study, we found no loss of expression and no mutation of *FEN1* in a large panel of human lung cancer cell lines. In addition, we detected a *FEN1* pseudogene. Microarray analysis showed that *FEN1* expression in SCLC cell lines was significantly increased compared to normal lung controls. These results suggest that alterations of *FEN1* through decreased expression or mutation are not likely to contribute to development of lung cancer.

There are possible explanations for the increased expression of FEN1. FEN1 is involved in DNA replication through its function in the processing of 5′ ends of Okazaki fragments in the lagging DNA strand (Bambara *et al*., 1997; Waga and Stillman, 1998) and thus increased expression of *FEN1* may reflect the increased proliferation rate of cancer cells compared to normal cells. In fact, the expression of Fen1 has been shown to be associated with proliferative populations. Warbrick *et al*. showed that the expression of Fen1 in serum-starved 3T3 cells was dramatically induced by re-feeding, indicating progression from quiescence into the cell cycle resulted in increase in Fen1 (Warbrick *et al*., 1998). Some cells can adapt to DNA alkylating agent exposure by increasing the expression of DNA polymerase β-pol mRNA, which also functions in the BER pathway (Chen *et al*., 1998). Thus, increased FEN1 expression may be a response caused by increased DNA damage in cancer compared to normal cells.

Hiraoka *et al*. performed FISH analysis of *FEN1* to determine its location and observed two signals, 11q12 and 1p22.2 (Hiraoka *et al*., 1995). They concluded 11q12 to be the location of *FEN1* by radiation-reduced hybrid cell analysis (Hiraoka *et al*., 1995). Since we discovered the *FEN1* pseudogene, the signal at 1p22.2 is likely to correspond to the location of the *FEN1* pseudogene. In fact, we found the *FEN1* pseudogene sequence in the NCBI contig sequence (NT_004686), which maps to chromosome region 1p22. Thus, we conclude that the *FEN1* pseudogene is located at 1p22.2.

In conclusion, our results suggest that loss of *FEN1* function is not likely to be involved in development of lung cancer. *FEN1* mRNA expression in SCLC cell lines was significantly upregulated compared to normal lung controls. FEN1 protein was abundantly expressed in all studied lung cancer cell lines but was expressed at lower levels in three of four normal lung epithelial cell controls. Further investigations such as search for alterations in other components of the DNA repair system are needed to clarify the underlying mechanisms of microsatellite alterations and other signs of genetic abnormalities found in lung cancer.

Abbreviations

SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; HNPCC, hereditary nonpolyposis colon cancer; RT–PCR, reverse transcriptase PCR; MSI, microsatellite instability; MMR, mismatch repair, BER, base excision repair.

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