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Over-expression and hypomethylation of *flap endonuclease 1* gene in breast and other cancers

Purnima Singh[1,†], **Ming Yang**[2,†], **Huifang Dai**[1], **Dianke Yu**[2], **Qin Huang**[3], **Wen Tan**[2], **Kemp Kernstine**[4], **Dongxin Lin**[2,*], and **Binghui Shen**[1,*]

[1]Department of Radiation Biology, City of Hope National Medical Centre and Beckman Research Institute, Duarte, California, USA

[2]Departments of Etiology and Carcinogenesis, Cancer Institute, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

[3]Division of Pathology, City of Hope National Medical Centre and Beckman Research Institute, Duarte, California, USA

[4]Division of Surgery, City of Hope National Medical Centre and Beckman Research Institute, Duarte, California, USA

Abstract

Flap endonuclease1 (FEN1) is a structure-specific nuclease best known for its critical roles in Okazaki fragment maturation, DNA repair and apoptosis-induced DNA fragmentation. Functional deficiencies in *FEN1*, in the forms of somatic mutations and polymorphisms, have recently been shown to lead to autoimmunity, chronic inflammation, and predisposition to and progression of cancer. In order to explore how FEN1 contributes to cancer progression, we examined *FEN1* expression using 241 matched pairs of cancer and corresponding normal tissues on a gene expression profiling array and validated differential expression by quantitative real-time PCR, and immunohistochemistry. Furthermore, we defined the minimum promoter of human *FEN1* and examined the methylation statuses of the 5' region of the gene in paired breast cancer tissues. We demonstrate that *FEN1* is significantly up-regulated in multiple cancers and the aberrant expression of *FEN1* is associated with hypomethylation of the CpG island within the *FEN1* promoter in tumor cells. The over-expression and promoter hypomethylation of *FEN1* may serve as biomarkers for monitoring the progression of cancers.

Keywords

Flap endonuclease 1; over-expression; promoter; hypomethylation

Introduction

The development of cancer involves altered expression of many genes during tumor formation and progression as a result of both genetic and epigenetic changes in the genome (1,2). The identification of genes that contribute to cancer outcome and progression is critical for the

[*]To whom correspondence should be addressed: Dongxin Lin, Departments of Etiology & Carcinogenesis, Cancer Institute, Chinese Academy of Medical Sciences, Beijing 100021, China. Phone: 8610-877-88491; Fax: 8610-677-22460; dlin@public.bta.net.cn or Binghui Shen, Department of Radiation Biology, City of Hope National Medical Centre and Beckman Research Institute, 1500 East Duarte Road, Duarte, California 91010, USA. Phone: 626-301-8879; Fax: 626-301-8280; bshen@coh.org.
[†]The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint first authors.

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development of appropriate therapy. Accumulating evidence shows that flap endonuclease 1 (FEN1) plays a pivotal role in the development of cancer and up-regulation of *FEN1* may be correlated with disease progression (3-5).

FEN1 is a structure-specific nuclease that is best known for its involvement in the maturation of Okazaki fragments during DNA replication and its efficient removal of 5′-flaps during long-patch base excision repair (LP-BER)(6-11). In addition to its 5′-flap endonuclease activity, FEN1 is also characterized as a 5′ exonuclease (EXO activity) (6,12) and a gap-dependent endonuclease (GEN activity) (13,14). In response to apoptotic stimuli, the GEN and EXO activities of FEN1 can be stimulated to promote apoptosis-induced DNA fragmentation. Considering its critical role in multiple DNA metabolic pathways, FEN1 is a key enzyme for maintaining genomic stability (15) and its deficiency results in predisposition to cancer (4) and rapid development of tumors (3).

FEN1 is widely expressed in mammalian cells and shows a high level of expression in testes, thymus, bone marrow and other highly proliferative tissues, in keeping with its role in DNA replication(16). In mouse embryonic fibroblasts, *FEN1* was shown to be up-regulated in a p53-dependent manner upon UV-C exposure (17). In addition, FEN1 protein is associated with proliferative cell populations (18-20). Its expression is up-regulated in metastatic prostate cancer cells(21), gastric cancer cells (22), neuroblastomas (23), pancreatic cancer (24) and lung cancer cell lines (25). *FEN1* is also highly expressed in comprehensive genome-wide tumor micro-array datasets for cell-cycle regulated genes (26). Recently, Lam et al. showed that *FEN1* is up-regulated in prostate cancer as compared to matched normal prostate and its expression increases with tumor progression(5), suggesting that FEN1 is a possible biomarker for patients at high risk for prostate cancer and a potential target for therapy.

To re-evaluate the expression of *FEN1* in various cancers and to understand the mechanism underlying such transcriptional activation, we performed a comprehensive analysis of *FEN1* expression in multiple cancers, and particularly in breast tissues, using a cancer profiling array that included paired normal/tumor specimens and *in vivo* immunohistochemistry in a breast progression cancer tissue array. We found that the nuclease gene was significantly over-expressed in cancer cells. We also tested whether DNA methylation plays a role in the regulation of *FEN1* expression, and identified a region of the *FEN1* promoter that was hypomethylated in the same cancer cells in which we observed gene over-expression.

Results

Expression of *FEN1* in tumor versus normal tissues

A cancer profiling array was used to examine *FEN1* expression in matched tumor tissues versus normal tissues. A general trend of *FEN1* over-expression was observed in all cancers examined (Supplementary Table 3, Figure 1A and 1B), except for in prostate cancer, where the difference was not statistically significant. An approximately 2.5-fold consistent increase in expression was found in breast and uterine cancer samples, while a 1–2-fold increase was found in other tumors, including colon, stomach, lung and kidney. In cases where metastatic samples were included, *FEN1* expression was significantly greater in the metastatic tissues as compared to the paired tumor tissues. This trend was observed in samples of breast, uterine, rectal, ovarian and colon cancers.

Of the 50 cases of breast tissues examined, 47 cases (94%) demonstrated greater *FEN1* expression than the matched normal tissues (Table 1, Figure 1C). In three breast cancer cases with matched metastatic samples, *FEN1* expression was higher in one metastatic tissue compared to the tumor tissues. The observed over-expression of *FEN1* in tumor samples from the cancer profiling array supports the hypothesis that increased expression of *FEN1* may be

associated with tumorigenesis. This is consistent with several other studies demonstrating FEN1 over-expression in various cancer tissues(5,21,22). The integrity of the samples on the array was confirmed by re-probing the array with a radio-labeled ubiquitin cDNA (data not shown). The *FEN1* cDNA did not hybridize with yeast total RNA, yeast tRNA, *E. coli* RNA, *E coli* DNA, poly r(A) and Cot-1DNA.

FEN1 expression at different stages of breast cancer

Immunohistochemical assessment of a breast cancer progression array showed that FEN1 was expressed in nuclei, with occasional cytoplasmic staining (Figure 2). We did not detect FEN1 in three normal breast tissues (Table 2); however, its expression was variable in tissues from different kinds of benign breast disease (BBD). In the 21 samples, including adenosis, fibrofatty tissue, fibroadenosis, adenosis with hyperplasia of epithelium and cystic hyperplasia, there was very low or no FEN1 immunohistochemical staining. The 11 samples with blunt duct adenosis, fibroadenosis with hyperplasia of epithelium, sclerosing adenosis, papillomatosis or papillomatosis with hyperplasia of epithelium tissues, were positive for FEN1 expression. Moreover, in infiltrating ductal carcinoma, FEN1 expression increased with corresponding disease stages and was highest in nonspecific infiltrating duct carcinoma III tissues. Among other three kinds of breast carcinomas, there was greater FEN1 expression in infiltrating lobular carcinoma than in medullary or mucous carcinoma. These findings suggest that the amount of FEN1 expression is directly correlated with the higher stages and grades of breast tumors.

A -458 bp to +278 bp sequence contributes to the basal *FEN1* promoter activity

To determine the location of the *FEN1* promoter, we studied the transcriptional activity of chimeric constructs of progressively 5-'deleted DNA fragments (-1821/+632). The various deletion fragments were cloned into a firefly luciferase-based reporter vector, pGL4.10, and subsequently co-transfected with a renilla-based reporter vector into HeLa cells to quantify the strength of the reporter activities driven by different deletion fragments (Figure 3). The empty pGL4.10 vector directed 0.46 ± 0.23 RLU of luciferase activity in HeLa cells. No significant transcriptional activity was detected with the -1821/+632 and +352/+2262 fragments (3.31 ± 0.32 RLU and 0.12 ± 0.02 RLU, respectively). 5' deletions of -1821/+632 fragment (1.5 ± 0.35RLU) ie., -1191/+632, -701/+632, -421/+632 and -281/+632 resulted in 5.7 ± 0.75, 5.8 ± 0.28, 3.5 ± 0.14 and 10.7 ± 1.05 RLU of luciferase acitivity, respectively. However, the maximum activity was shown by the –458/+278 fragment (32.54 ± 3.81 RLU). The –458/+278 fragment has been shown to be the minimum promoter to drive expression of the mouse *FEN1* (27), which suggests that the homologous fragment is also the minimum promoter to drive human *FEN1* expression.

FEN1 methylation analysis

The mechanism responsible for the regulation of *FEN1* expression is largely unknown. *In silico* analysis indicated that there are two CpG islands in the *FEN1* promoter (Figure 4A). To elucidate the methylation status of the CpGs in these islands, we performed bisulfite sequencing on samples from six paired breast cancer and normal tissues. We detected methylated DNA only in CpG island 2 in normal breast tissues (Figure 4B). In contrast, breast tumors showed no methylation of either CpG island. Furthermore, we analyzed endogenous *FEN1* expression by real-time RT-PCR in the same six paired breast cancer and normal tissues (Figure 4C). *FEN1* mRNA was expressed at different levels in the specimens tested, and there was a good correlation with the observation that *FEN1* is significantly up-regulated in tumors. These results suggest that hypomethylation of the *FEN1* promoter in tumors is associated with increased expression of *FEN1*.

Discussion

As a multifunctional nuclease, FEN1 is involved in DNA replication, repair and apoptosis. Although it is up-regulated in multiple types of cancer(21-25), it is unclear whether *FEN1* expression is also increased in breast and other major cancers. Therefore, we investigated its mRNA abundance in matched tumor and normal samples using a cDNA array. Consistent with previous observations, *FEN1* was over-expressed in 178 tumor cases as compared to normal tissues in all 236 primary tumor samples (75.42%) examined. However, we did not detect up-regulation of *FEN1* in prostate cancer, which may be due to the small sample size (only 4 cases). *FEN1* expression has been reported to be induced for DNA replication during cell proliferation (20). Thus, increased expression of *FEN1* may reflect the increased proliferation rate of cancer cells. Moreover, when cancer cells are exposed to DNA alkylating agents, some cells can adapt to these exposures by increasing the expression of DNA polymerase β, a core enzyme in the BER pathway (28). Because FEN1 is involved in the BER pathway, increased expression of *FEN1* may also be a response to increased DNA damage in cancer cells. Interestingly, we found that, in female cancers, not only the ratio of samples with *FEN1* over-expression was the highest (86.92%), but also the amount of *FEN1* over-expression. It has been reported that FEN1 could interact directly with estrogen receptor-alpha (ERα), enhance the interaction of ERα with ERE-containing DNA and influence estrogen-responsive gene expression (29,30). Conversely, *FEN1* expression can also be regulated by estrogen in the uterus (31). These findings suggest that *FEN1* over-expression may be precisely regulated by hormones in female cancers.

In this study, we found greater *FEN1* RNA expression in breast cancer than in other cancer types. Based on this observation, we analyzed endogenous FEN1 protein expression by immunohistochemical staining in a breast cancer progression tissue array. Consistent with the RNA expression results, FEN1 was up-regulated in tissues from BBDs with atypical hyperplasia and several kinds of breast cancer tissues. It has been shown that atypical hyperplasia has a relative risk of 4.24, proliferative disease without atypia has a relative risk of 1.88, and nonproliferative lesions have a relative risk of 1.27 (32). Interestingly, our results also show that *FEN1* expression increases with an increase in relative cancer risk in BBD tissues. Moreover, in agreement with previous studies that examined the role of *FEN1* expression in prostate cancer (5), we found that increased *FEN1* expression was associated with tumor dedifferentiation in infiltrating ductal carcinoma. These results suggest that *FEN1* might be a potential tumor marker for selecting patients at high risk of progression.

To determine the underlying mechanism of the differential transcriptional regulation of *FEN1* between tumor and normal tissues, we investigated the possibility of epigenetic regulation of *FEN1* expression via methylation. We found that over-expression of *FEN1* was associated with hypomethylation within CpG island 2 of the *FEN1* promoter in breast cancer. Genome-wide hypomethylation and regional hypermethylation of certain genes have been observed in several human cancers, including breast cancer (33,34). The former may lead to activation of genes such as oncogenes and expression of provirus sequences; the latter may result in gene silencing of tumor suppressor genes (35). Therefore, it has been proposed that hypomethylation and hypermethylation in cancer are independent processes, which target different programs at different stages in tumorigenesis (36). Accumulating data have led to the hypothesis that hypomethylation plays a role in activating certain genes required for cancer progression and metastasis of breast cancer (37,38). In agreement with these studies, we also examined the role of aberrant DNA hypomethylation in *FEN1* gene expression in breast cancer progression.

In conclusion, our results suggest that *FEN1* promoter hypomethylation may contribute to its over-expression in poorly differentiated carcinomas. To the best of our knowledge, this is the

first study to show how methylation of *FEN1* promoter region affects gene expression in breast cancer. Furthermore, the elevated expression of *FEN1* may serve as a useful molecular marker for predicting prognosis and as a target for therapy.

Materials and Methods

Cancer Profiling Array I

To compare the expression of *FEN1* in human tumors and corresponding normal tissues, we used the Cancer Profiling Array I (BD Biosciences Clontech Inc., Palo Alto, CA). This array includes normalized cDNAs from tumors of 241 individual patients and corresponding normal tissues, including breast, uterus, ovary, cervix, lung, kidney, stomach, colon, rectum, small intestine, pancreas and prostate as well as 12 cDNAs from metastases corresponding to 12 of the tumor/normal pairs. ^{32}P-labelled cDNA probes were synthesized from human *FEN1* or ubiquitin control cDNA using a random primer-labeling kit (New England Biolabs, Beverly, MA) followed by probe purification on CHROMA SPIN+STE-100 columns (Clontech). The *FEN1* fragment corresponding to 463 bp-734 bp, was used to probe the filter. Hybridization of the Cancer Profiling Array I with *FEN1* probes and washing of the array were done according to the manufacturer's recommendations (Clontech). The hybridized arrays were then exposed to phosphorimaging screen, scanned with a Typhoon Phosphorimager and analyzed using ImageQuant 1.2 software (Amersham Biosciences, Sunnyvale, CA). The array was then stripped and hybridized with the human ubiquitin cDNA probe to confirm the integrity of the samples on the array. Statistical analysis of the cancer-profiling array was done using GraphPad Prism 2.01 software and two-tailed paired t-test ($P \leq 0.05$).

Immunohistology

For *in situ* and *in vivo FEN1* expression analysis, a breast carcinoma progression array (Cybrdi, Frederick, MD) was used. Immunohistochemical staining was performed using a monoclonal anti-FEN1 antibody (NCL-Fen-1; Novocastra, Newcastle upon-Tyne, UK) according to the manufacturer's instructions. Staining was semi-quantitatively assessed by the pathologist at the City of Hope Pathology Core Facility, and two criteria of *FEN1* expression were scored: the intensity on a 0–3 scale (0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive), and the percentage of positively-stained target cells (0, no cells; 1, less than 10% of the cells; 2, 11–50% of the cells; 3, 51–80% of the cells; 4, more than 80% of the cells). Finally, a composite score was obtained by multiplying the values of the mean staining intensity and the percentage of FEN1-positive cells (0–1, negative; 1–2, weakly positive; 2–3, moderately positive; ≥3, strongly positive).

Promoter reporter constructs

Specific primer pairs (Supplementary Table 1) with *Sac*I and *Nhe*I restriction sites were used to amplify multiple deletion fragments spanning 5′ flanking region of *FEN1* (from –1821 bp to +2662 bp, relative to the transcription start site) from human genomic DNA (Clontech) using Hot Start Taq (Qiagen, Valencia, CA). The PCR products were then digested with *Sac*I and *Nhe*I (New England Biolabs) and ligated into an appropriately digested pGL4.10 vector (Promega, Madison, WI) containing the firefly luciferase gene as a reporter. The constructs were designated as pGL4-FP1 to pGL4-FP9 (Supplementary Table 1). Restriction analysis and complete DNA sequencing confirmed the orientation and integrity of the inserts.

Transient Transfection and Luciferase Assays

Transient transfection of HeLa cells was performed using LipofectAmine (Invitrogen, Carlsbad, CA). Cultured cells were cotransfected with 1μg of one promoter construct and 0.1 μg of pGL4.74 [*hRluc*/TK] vector, a renilla luciferase control reporter vector (Promega,

Madison, WI) that was used as an internal control to normalize the activities of the experimental reporters. After transfection in serum-free medium, the cells were allowed to recover in serum-containing medium. The cells were lysed 48 hrs post-transfection and assayed for promoter activity using the Dual–Luciferase Reporter Assay System (Promega, Madison, WI). The luciferase activity was measured using a Fluoroskan Ascent FL luminometer (Thermo Electron Corp., Waltham, MA). Luciferase values (relative light units, RLU) were calculated by dividing the firefly luciferase activity by the renilla luciferase activity. The assay was conducted three times in duplicates.

Tumor Specimens

DNA methylation analysis and RNA preparation were performed on six paired specimens of breast tumors and corresponding normal tissues selected from the archives of Cancer Hospital, China. Histopathological diagnosis of the tumors was performed according to the World Health Organization classification (Supplementary Table 2). Patients with metastasized cancer from other organs were excluded. This study was approved by the institutional review board of the Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China).

Methylation Analysis

The 5′ flanking region, exon1 and the intron of human *FEN1* were analyzed by Methprimer software (http://www.urogene.org/methprimer/index1.html). Two CpG islands, CpG1-297 bp and 24 CpG dinucleotides and CpG2-399 bp and 37 CpG dinucleotides, were detected. Paired normal and breast caner genomic DNA samples were modified by bisulfite reaction using the EZ DNA Methylation Kit (Zymo Research, Orange, CA). Up to 2 μg of genomic DNA was used for conversion with the bisulfite reagent. Approximately 80 ng of bisulfite-converted DNA was used as a template for each PCR analysis. Primers for bisulfite sequencing were designed using the Methprimer software. The primer pairs CpG-1F 5’-AGTTGAGAAATTTAAGGAGT-3’, CpG-1R 5’-CTCCAAAAAAAACAAAATCT-3’ and CpG-2F 5’-GAGGGATTGGTTGTTATGAGAGTAG-3’, C p G-2R 5’-ACCCCATAAAATAAAACTTATTACC-3’ were used to amplify the target CpG islands with Hot Start Taq polymerase (Qiagen). The amplified fragments were cloned into the pSC-A vector (Stratagene). Individual clones were sequenced and compared with the original sequence to calculate the percentage of methylation.

Quantitative Real-time PCR

Total RNA was isolated from paired tissues and converted to cDNA using an oligo $(dT)_{15}$ primer and Superscript II (Invitrogen). Quantitation of relative gene expression for *FEN1* and *β-actin* as an internal reference gene was carried out using the ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA) in triplicates, based on the SYBR-Green method . The primers used for *FEN1* were 5′-CTGTGGACCTCATCCAGAAGCA-3′ and 5′-CCAGCACCTCAGGTTCCAAGA-3′; and for *β-actin* were 5′-GGCGGCACCACCATGTACCCT-3′ and 5′-AGGGGCCGGACTCGTCATACT-3′. The PCR specificity was confirmed by dissociation curve analysis and gel electrophoresis. The expression of individual *FEN1* measurements was calculated relative to expression of *β-actin* using a modification of the method described by Lehmann and Kreipe (39).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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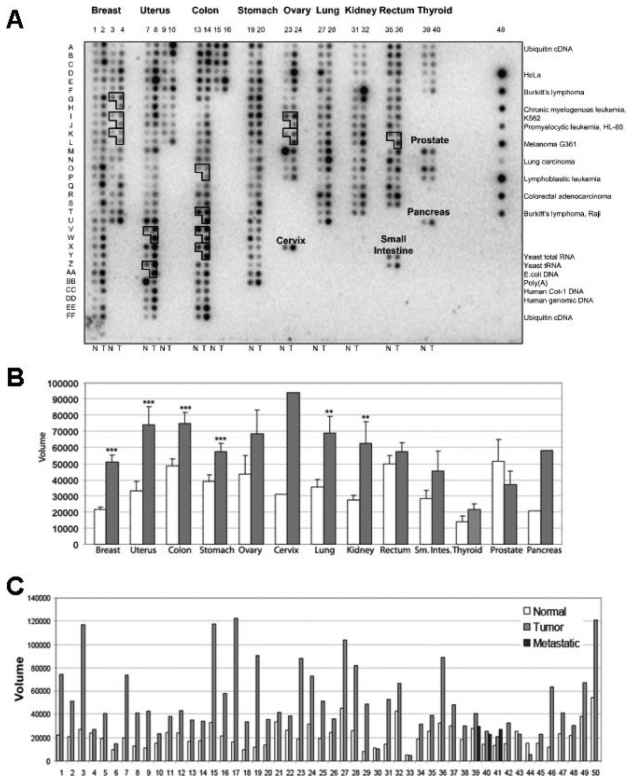

Figure 1.

Expression of *FEN1* in matched normal and tumor tissues. (A) Cancer Profiling Array I contains cDNA samples from 241 paired human tumors (T) and normal (N) tissue from individual patients. The boxed samples represent normal, tumor and metastatic samples form the same individual. The array was hybridized with a ^{32}P-labelled cDNA probe for *FEN1*. (B) The data from the Cancer Profiling Array I were quantified using ImageQuant software. The mean volumes ± SE are shown. Asterisks indicate a statistically significant difference between the matched normal (white bars) and tumor tissues (grey bars) as determined by two-way paired *t*-tests ($P \leq 0.05$). A statistically significant greater amount of *FEN1* expression was found in breast tumor tissue (~2.4 fold, $P < 0.0001$, n = 50), uterine tumor tissue (~2.3 fold, $P = 0.0006$, n = 42), colon tumor tissue (~1.5 fold, $P < 0.0001$, n = 35), stomach tumor tissue (~1.5 fold, $P = 0.0005$, n = 28), lung tumor tissue (~1.9 fold, $P = 0.0066$, n=21) and kidney tumor tissue (~2.3 fold, $P = 0.0063$, n = 20), compared to matched normal tissues. (C) Expression of *FEN1* in 50 matched samples of normal breast and tumor tissue. Out of all 50 cases, 47 cases exhibited greater *FEN1* expression in tumor tissue compared with the matched normal tissue. Three of the 50 cases had a matched metastatic sample (cases 39, 40, and 41), and the level of *FEN1* expression in only one of them was greater than in the matched tumor tissue.

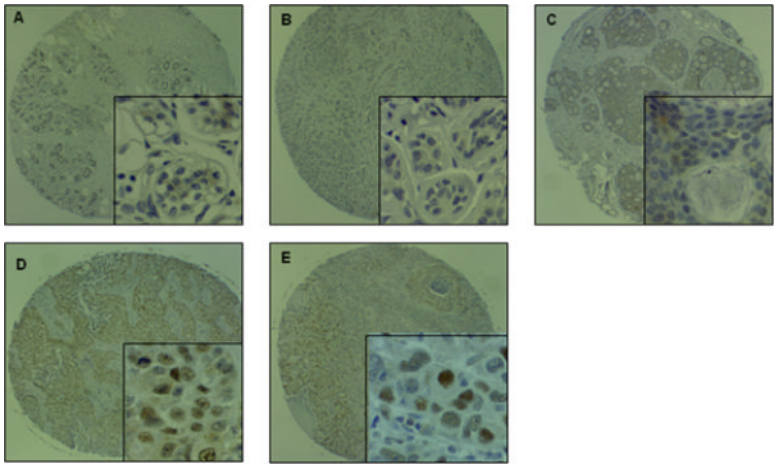

Figure 2.
Immunohistochemical analysis of a breast cancer progression array using anti-FEN1 antibody. (A) normal, (B) benign breast tissue with adenosis, (C) breast tissue with ductal carcinoma *in situ*, (D) breast tissue with poorly differentiated carcinoma, (E) breast tissue with poorly differentiated carcinoma. Reduced from ×10 (A to E) and × 40 (inserts).

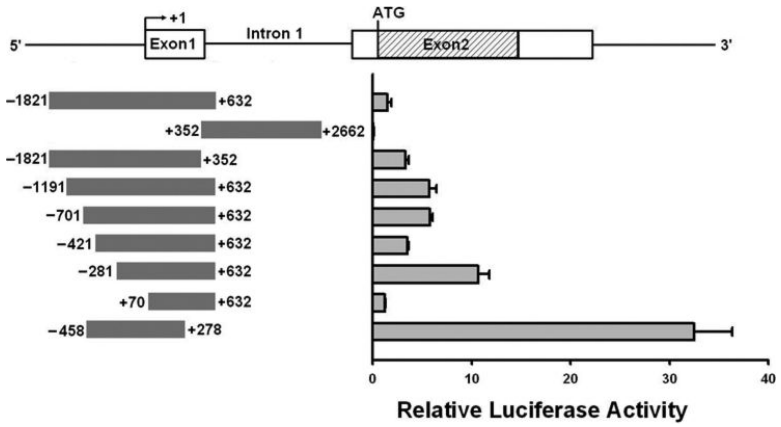

Figure 3.

A–458 and +278 fragment confers basal activity of the *FEN1* promoter. The transcriptional start site (+1), exon 1, intron 1 and exon 2 of the *FEN1* gene are shown. On the left, below the gene diagram, the various 5′ or 3′ deletion constructs and their 5′ and 3′ positions with respect to the transcriptional start site are depicted. On the right, below the gene diagram, the normalized luciferase activity of the constructs is given. Fold increases were measured by defining the activity of the empty pGL-4.10 vector as 1. Data are shown as the mean fold increases ± SE from 3 independent transfection experiments, each performed in duplicate.

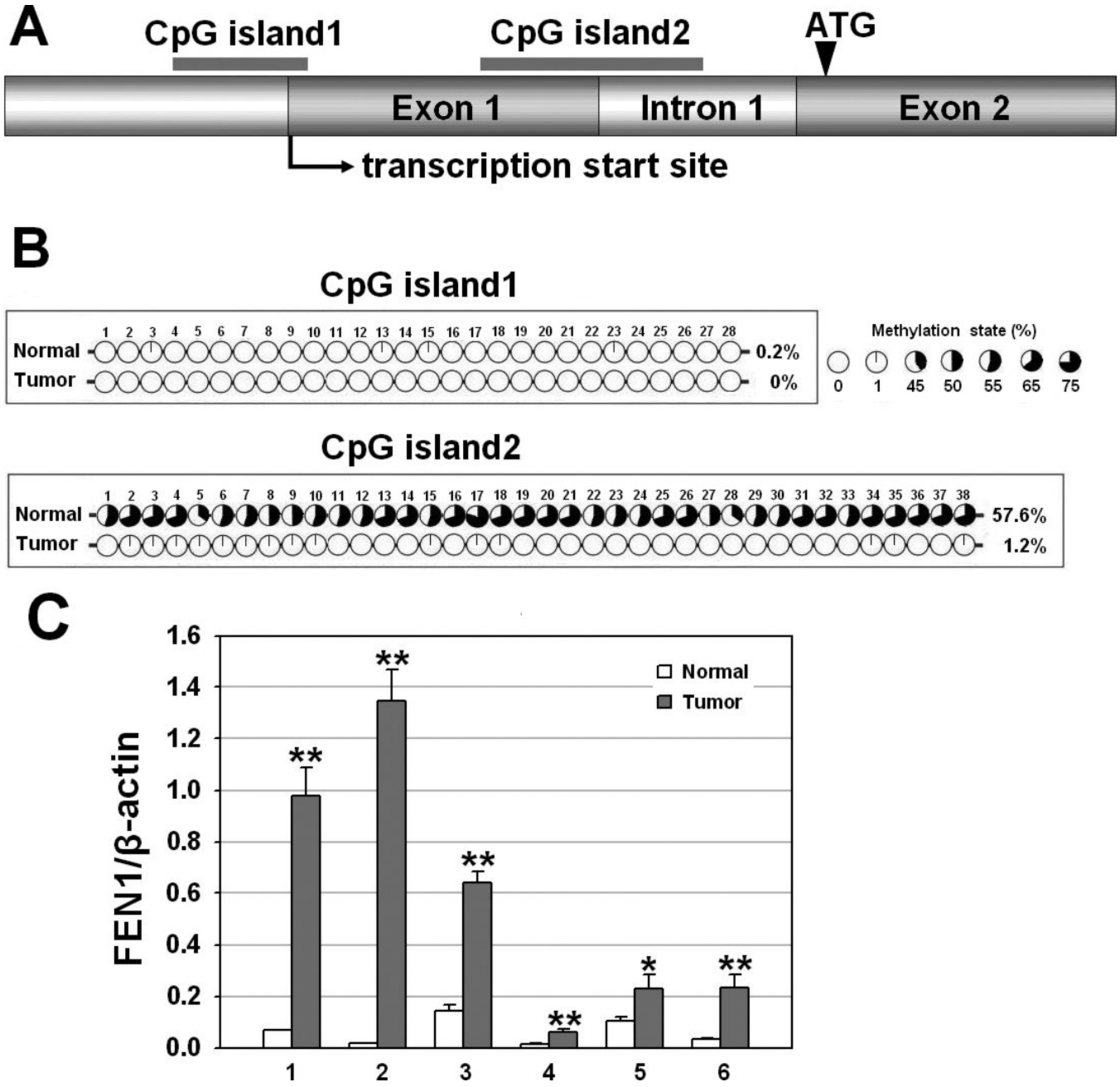

Figure 4.

FEN1 promoter methylation analysis and mRNA expression in paired breast cancer tissues. (A) CpG islands within the *FEN1* promoter were analyzed by Methprimer. Two CpG islands, CpG islands 1 and 2, were detected. CpG islands 1 and 2 are within the characterized promoter –458 bp to +278 bp region. (B) Sodium bisulfite sequencing analysis of the *FEN1* promoter in paired normal and tumor tissues from patients with breast cancer. Six pairs of matched normal and tumor samples were sequenced. The methylation levels of each CpG dinucleotide located in CpG islands 1 and 2 are indicated. (C) Levels of *FEN1* mRNA expression in 6 normal and matched breast cancer tissues are presented as mean ± SE normalized to *β-actin*. **P* < 0.05, ***P* < 0.01.

Table 1

Clinical information and fold change in *FEN1* expression in the 50 cases of matched normal and breast tumor tissue

#	Age	Pathology	Fold change in FEN1[a]
1	39	noninfiltrating intraductal carcinoma	↑ 3.31
2	78	infiltrating ductal carcinoma	↑ 2.45
3	49	infiltrating ductal carcinoma	↑ 4.31
4	52	tubular adenocarcinoma	↑ 1.11
5	66	infiltrating ductal carcinoma	↑ 2.08
6	65	lobular carcinoma	↑ 1.52
7	44	infiltrating ductal carcinoma	↑ 3.74
8	33	infiltrating ductal carcinoma	↑ 3.26
9	40	infiltrating ductal carcinoma	↑ 3.85
10	49	infiltrating ductal carcinoma	↑ 1.55
11	41	lobular carcinoma	↑ 1.57
12	50	infiltrating ductal carcinoma	↑ 1.80
13	61	lobular carcinoma	↑ 2.09
14	64	infiltrating ductal carcinoma	↑ 1.97
15	40	noninfiltrating intraductal carcinoma	↑ 3.56
16	50	infiltrating ductal carcinoma	↑ 2.69
17	44	infiltrating ductal carcinoma	↑ 7.47
18	52	infiltrating ductal carcinoma	↑ 3.45
19	47	infiltrating ductal carcinoma	↑ 7.83
20	59	infiltrating ductal carcinoma	↑ 2.56
21	50	infiltrating ductal carcinoma	↑ 1.25
22	68	infiltrating ductal carcinoma	↑ 1.47
23	63	tubular adenocarcinoma	↑ 4.63
24	44	fibrosarcoma	↑ 2.32
25	49	infiltratin globular carcinoma	↑ 2.67
26	64	infiltrating ductal carcinoma	↑ 1.47
27	50	infiltrating ductal carcinoma	↑ 2.29
28	39	mixed lobular-ductal carcinoma	↑ 3.17
29	66	infiltrating lobular carcinoma	↑ 5.99
30	65	infiltrating lobular carcinoma	↓ 0.91
31	61	infiltratin globular carcinoma	↑ 3.70
32	38	infiltratin globular carcinoma	↑ 1.55
33	58	lobular carcinoma	↓ 0.90
34	64	infiltrating ductal carcinoma	↑ 1.68
35	46	tubular adenocarcinoma	↑ 1.53
36	41	infiltrating ductal carcinoma	↑ 2.73
37	60	lobular carcinoma	↑ 1.61
38	62	infiltrating ductal carcinoma	↑ 1.63
39	71	infiltrating ductal carcinoma	↑ 1.44

#	Age	Pathology	Fold change in FEN1[a]
40	52	infiltrating ductal carcinoma	↑ 1.78
41	57	infiltrating ductal carcinoma	↑ 1.56
42	61	infiltrating ductal carcinoma	↑ 2.22
43	40	infiltrating ductal carcinoma	↓ 0.90
44	48	infiltrating ductal carcinoma	↓ 0.37
45	45	lobular carcinoma	↑ 1.48
46	47	medullary carcinoma	↑ 5.45
47	60	infiltrating ductal carcinoma	↑ 1.77
48	71	infiltrating ductal carcinoma	↑ 1.41
49	53	mucinous adenocarcinoma	↑ 1.76
50	42	infiltrating ductal carcinoma	↑ 2.22

[a]The fold-increase (↑) or decrease (↓) in *FEN1* expression in the tumor tissues is shown for each individual pair

Table 2

Immunohistochemical analysis of a breast cancer progression tissue array

Tissue type	Mean staining intensity[a]	Mean positively-stained cells (%)	Semi-quantitative scores[b]	Positively-stained tissue spots (%)
Breast tissue	0.00	0.00	–	0 (0/3)
Adenosis and Fibrofatty tissue	0.00	0.00	–	0 (0/6)
Fibroadenosis and Adenosis with hyperplasia of epithelium	0.42	0.84	–	30.8 (4/13)
Cystic hyperplasia	0.50	2.50	–	50 (1/2)
Blunt duct adenosis and Fibroadenosis with hyperplasia of epithelium	1.50	2.50	+	100 (4/4)
Sclerosing adenosis	0.75	13.75	+	50 (4/4)
Papillomatosis and Papillomatosis with hyperplasia of epithelium	1.00	7.50	+	66.7 (2/3)
Intraductal carcinoma	0.00	0.00	–	0 (0/3)
Nonspecific infiltrating duct carcinoma I	1.13	1.75	+	75 (3/4)
Nonspecific infiltrating duct carcinoma II	1.19	6.40	+	62.5 (5/8)
Nonspecific infiltrating duct carcinoma III	2.42	45.80	+++	83.3 (5/6)
Infiltrating duct carcinoma with lymph node metastesis	0.75	3.30	–	50 (3/6)
Medullary carcinoma	0.66	5.00	–	33.3 (1/3)
Mucous carcinoma	1.33	10.00	+	66.7 (2/3)
Infiltrating lobular carcinoma	1.75	23.30	+++	100 (3/3)

[a] The intensity was graded as absent (0), weakly positive (1), moderately positive (2) or strongly positive (3) compared to normal breast tissue.

[b] A composite score was obtained by multiplying the values of the mean staining intensity and the mean percentage of FEN1-positive cells.