CANCER GENOMICS & PROTEOMICS 5: 287-292 (2008)

Genomic Changes of the 55 kDa Subunit of DNA Polymerase ε in Human Breast Cancer

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Abstract. *Background: DNA polymerases (Pols) represent potential candidates for cancer genes because of their central functions in DNA metabolism. Defects of some DNA Pols have shown cancer associations, but data on DNA polymerase (Pol) ε is limited. Materials and Methods: Twenty-four human breast cancer DNA samples and four control DNA samples were examined for possible mutation in the entire coding region of the 55 kDa small subunit of the human DNA Pol ε gene using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis of the DNA and sequence analysis. In addition, 20 control DNAs were studied with PCR-SSCP for the end of intron 18 and exon 19 region. Results: An AATT deletion was found at one location in intron 18 in 2 out of the 24 breast cancer cases (8%), but in none of the control cases. In addition, a single base transition was found in the cancer DNAs in intron 14, but the same changes were also found in the control DNAs, suggesting polymorphism. Conclusion: Specific changes might occur in the 55 kDa small subunit DNA sequence of DNA Pol ε in breast cancer. The deletion at the region of intron-exon junction may not affect the protein code, but could potentially influence splicing efficiency and expression levels, possibly impairing the function of Pol ε DNA.*

DNA polymerases (Pols) have a central role in the maintenance of DNA (1). At least 14 Pols have been identified in the mammalian cell, but only three of these, Pols α, δ and ε, are considered to perform the bulk of DNA synthesis during replication (1). These Pols are structurally related enzymes of family B that are all essential for nuclear DNA replication and also fulfil additional roles in DNA repair and cell cycle control (2). Among the Pols, polymerase (Pol) ε is well conserved in sequence and subunit composition among eukaryotes. Human Pol ε located on chromosome 14 is composed of four subunits, a large catalytic subunit of 261 kDa and three associated subunits of 55, 17 and 12 kDa (3). An antibody that inhibits Pol ε activity reduced DNA synthesis when injected into human fibroblasts (4), and immunodepletion of Pol ε impaired the elongation step of DNA replication in *Xenopus* egg extracts (5). Human Pol ε has been implicated both in nucleotide-excision repair and long patch base excision repair (6-8), and specifically in yeast, in leading strand synthesis (9). The 55 kDa small subunit of Pol ε, as a DNA pol associated B subunits protein (10), has been implicated in stabilization of the catalytic subunit, mediation of the protein-protein interaction, and cell-cycle control.

A recent study has associated defects of mitochondrial Pol with premature aging (11). Links to cancer may be associated with other Pols, especially with Pol β. Pol β has a critical role in base excision repair, and functional impairment can lead to development of the mutator phenotype (12). Deletions of chromosome 8p are relatively frequent events associated with several forms of cancer (13), and Pol β is located in that region. In colorectal cancer, a large number of base deletions in mRNA derived cDNA have been reported in Pol β (14). These changes occurred more frequently in cancer tissue (in 5 out of 6 cases) than in normal mucosa or placental tissue (14). Corresponding changes have been reported by Dobashi *et al.* in prostate cancer (15), and Matsuzaki *et al.* (16) in human bladder cancer. The Pol β error spectrum in the *Adenomatous Polyposis Coli* (APC) gene *in vitro* contains human colon tumor mutational hotspots, indicating that inaccurate DNA synthesis by Pol β may be the cause of mutations that underlie the development of colon cancer (17).

Abbreviations: PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; Pol, polymerase; BAC, bacterial artificial chromosome.

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Key Words: Breast cancer, DNA polymerase epsilon (ε), genomic changes.

Other Pols have also been implicated in tumorigenesis. Point mutations have been detected in the Pol δ in colon cancer cell lines, and in primary human colon cancer (18). Mutations in the Pol η gene underlie the cancer-prone *Xeroderma pigmentosum* variant syndrome (XP-V) (19, 20). XP-V cells are deficient in the bypass DNA synthesis of DNA strands after UV irradiation.

As a step towards better understanding of the potential role of Pol ε in carcinogenesis, the gene coding for the exon regions of the 55 kDa subunit of human DNA pol ε was screened for variations in breast cancer samples. We now know that breast cancers differ in different populations (21, 22). These differences seem to be associated with the subtle evolutionary genetic differences of human populations (23, 24) but at the moment we do not know how these differences influence the characteristics of breast cancer.

Materials and Methods

Studied samples. Twenty four human breast tumor samples, and four control samples (lymph node, placenta) and 20 extra control samples (lymph node), all collected from the sample files of the Turku University Central Hospital, were used in this study. DNA was extracted from paraffin embedded material, except for the placental sample which was fresh, using standard methods (25) with minor adjustment.

PCR amplification. The 19 sets of intronic primers shown in Table I were designed to amplify the entire coding region of the 55 kDa subunit of human Pol ε (26). The SP6 promoter sequence, GACACTATAGAATAC and T7 promoter sequence, CGACTCAC TATAGGG, were attached to the 5' end of each upstream and downstream primer respectively. The 35 cycles of polymerase chain reaction (PCR), each cycle consisting of denaturing for 1 min at 94˚C, except with exons 1, 11 and 12 at 95˚C, annealing at different temperatures (Table I) for 1 min and extension for 1 min at 72˚C, were performed in a Perkin Elmer Cetus DNA Thermal Cycler 480 (Perkin Elmer, Norwalk, Connecticut, USA). A second pair of Cy^5 labelled primers, (SP6 5': TTTAGGTGACACTATAGAATAC and T7 5':GTAATACGACTCACTATAGGG) were used for subsequent secondary PCR and to produce Cy^5 labeled PCR products. The 30 cycles included 30 sec denaturation at 94˚C , except exon 1 at 96˚C, 30 sec annealing at 55˚C and 30 sec extension at 72˚C. The PCR products were electrophoresed on 2.5% agarose gel.

Single strand conformation polymorphism (SSCP) analysis. The Cy^5 labelled PCR products were mixed with an equal volume (4.5 μl) of denaturing solution containing 100% deionized formamide and 0.05% bromophenol. The mixture was heated at 95˚C for 4 min, thereafter chilled on ice. Two undenatured samples (one from the tumors, one from controls, no heating at 95˚C) were added to each run. A total of 2.5 μl of the mixture was loaded on a 6% polyacrylamide gel (acrylamide:bisacrylamide, 99:1) in an ALFexpress II with Cool kit (Pharmacia Biotech, Uppsala, Sweden). The running conditions for the gel electrophoresis were 35 W for 10 h at 12˚C with external cooling. The SSCP data were collected by ALFwin software and analyzed by a fragment analyser 1.02 (Pharmacia Biotech).

Sequence analysis. The samples displaying variant bands on SSCP were additionally analyzed by sequencing. The DNA samples were amplified by PCR as described above. The amplification products were purified with a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Uppsala, Sweden). The purified PCR product was sequenced using a Thermo Sequenase Cy™ 5 Dye Terminator Kit according to protocols provided by the manufacturer (Amersham Biosciences). The sequencing products were electrophoresed on 6% PAGE gel on an ALFexpress II DNA sequencer (Pharmacia Biotech). The sequence data were collected by ALFwin software and analyzed by a sequence Analyser 2.00 (Pharmacia Biotech). The sequencing results were compared with chromosome 14 genomic sequence data from bacterial artificial chromosome (BAC)-clones (27) and 55 kDa subunit of human Pol ε sequencing data from HeLa cells (26).

Results

The comparison with the BAC-clone data showed fewer sequence abnormalities than the comparison with the HeLa cell DNA data suggesting that HeLa cell data as a reference is degenerated by the progressive development of cancer associated genomic changes. A SSCP bandshift was observed when screening exon 19 in 2 out of the 24 breast tumors. Direct sequencing revealed an AATT deletion at nucleotides 25115-25118 in intron 18, 87 bp before the start of exon 19, in the two cases of breast cancer. Similar deletions were not found in the control DNAs (Figure 1).

To confirm that the observed specific Pol ε deletion was breast cancer related and not a polymorphism in normal tissues, 20 extra control samples (lymph node) were analysed by SSCP. All the samples showed the wildtype SSCP band pattern, with the exception of one sample in which the same bands had a slightly different position on the gel. When the latter sample and 4 randomly chosen normal DNAs were sequenced no changes were found.

A polymorphic DNA alteration was detected in the samples by the primer set 14 (exon 14). Ten out of the 24 breast tumor and 3 out of the 4 control samples showed a bandshift in intron 14. Sequencing results showed this to be a G-A transition at nucleotide 35481 (Figure 2). Because of overlap, a corresponding change was also present when primer 15 was used. Table II presents sequence differences between the present study and the genomic chromosome 14 reference sequence (27). These differences from published sequences probably demonstrate differences in genetic background of the source DNA.

Discussion

The importance of studying the genomic variation in the 55 kDa unit of polymerase ε is supported by the recent finding by Jaszczur *et al*. that the corresponding unit in yeast, the non-catalytic Dpb2p, contributes to the fidelity on DNA replication (28). Variations in this part of the enzyme may expose the genome to increased mutation pressure which may affect

Table I. *Intronic primer sets for amplification of exons 1-19 for the 55 kDa small subunit of the DNA pol ε gene.*

Exon	Position	Sequence of primer[a]	AT[b]	Size of product (bp)
1	upstream	5'SP6[c]-AGTTTCCCGGCAGCAAAG	60	350
	downstream	5'T7[d]-CTCTCGCCCTTCAAGCTG		
2	upstream	5'SP6[c]-TGACCTGCCTCATTTTTCCT	63	307
	downstream	5'T7[d]-GGGAAGAAATGCTACCATGC		
3	upstream	5'SP6[c]-GGGAAAAGAGCTGTCTGTGG	61	222
	downstream	5'T7[d]-AACACCTCCAAGGAGGAAAAA		
4	upstream	5'SP6[c]-GCCTGGCCTGAGAAAATGT	65	320
	downstream	5'T7[d]-AGGTGAGCCAAAAGTGAAGC		
5	upstream	5'SP6[c]-GCTTCACTTTTGGCTCACCT	63	308
	downstream	5'T7[d]-GACCAACTTTGGACCACAAAA		
6	upstream	5'SP6[c]-TGTTTCATCAGGGCACTATTTT	55	321
	downstream	5'T7[d]-CATGCAGCCCATCCAGTTA		
7	upstream	5'SP6[c]-AAAGTTTTGTTTCCTGAAAGAATATA	50	223
	downstream	5'T7[d]-TCATCAATCCAAGGCCAAA		
8	upstream	5'SP6[c]-TTTTTGTCAACGGTTTTCATTG	55	276
	downstream	5'T7[d]-CATTCCTACACCAGCAACATAGA		
9	upstream	5'SP6[c]-GAAGCCTTGTTGGACTCAAAA	63	285
	downstream	5'T7[d]-TTTGCCAAAAATATGCTCTTC		
10	upstream	5'SP6[c]-GCCTGGCATTATTGCTTTGT	58	242
	downstream	5'T7[d]-CCTGCCAGTATTACCAAATTCC		
11	upstream	5'SP6[c]-TTCATTAGATACAAAGAAGCTAAATGT	48	330
	downstream	5'T7[d]-AAAGAGTTCAAGAAAGAAGAAAAAA		
12	upstream	5'SP6[c]-CATGGTTAAAGGGTTTTTAACTTGA	48	328
	downstream	5'T7[d]-TTGCATAAAATGCCATAAAAT		
13	upstream	5'SP6[c]-CATTTTATGCAAATTTAATTCAATACC	52	175
	downstream	5'T7[d]-TTTTTTAAGTAAAACGGTGAAAA		
14[e]	upstream	5'SP6[c]-TGAATGGTGTTGAATTTTGCT	56	218
	downstream	5'T7[d]-TTCAGCAAGTGGTGGCCTA		
15[e]	upstream	5'SP6[c]-GGATCCTGGATTTGGTTCC	56	289
	downstream	5'T7[d]-CCCCCACACCTGGCTAAT		
16	upstream	5'SP6[c]-AATGTGGGAATGGGGTTGT	53	277
	downstream	5'T7[d]-GCCCCATGTAAGGCAGTTT		
17	upstream	5'SP6[c]-TGCAAATGAACTGAAGTTTGCT	52	357
	downstream	5'T7[d]-GCATATTTTTACTTCGACAATAATCTT		
18	upstream	5'SP6[c]-GAGCATTCACTTACAGGCATTTT	60	244
	downstream	5'T7[d]-GGGCCACTGTAGACATTTTCA		
19	upstream	5'SP6[c]-AACAGTATGTATGTATTGACCTGTTT	56	294
	downstream	5'T7[d]-CAAATTTAAGCAGAACATCCTAA		

[a]Primer sequences correspond to the genomic DNA report in ref. 22; GenBank accession number AH010907; [b]AT, Annealing temperature; [c]SP6, fixed SP6 promoter sequence GACACTATAGAATAC attached to 5' end of each upstream primer; [d]T7, fixed T7 promoter sequence CGACTCACTATAGGG attached to 5' end of each downstream primer; [e]Primers 14 and 15 products are partly overlapping.

proofreading. It is also important to realize that the human genome has a slow tendency to change, and that the corresponding potential protein change is corrected by selection within the population in conditions that occur during the reproductive period of an individual, but less so thereafter, at the ages when the bulk of cancers emerge (29).

Potentially the observed deletion in the present study has an association with the origin or progression of breast cancer, but may also be a coincidental association with an unusual polymorphism in this patient population. The normal (wild type) sequence was also found in the tumor specimens from these two cases, possibly reflecting heterozygotic alleles or non-tumor tissue present in the original sample. The latter finding may suggest that the change was associated with neoplastic DNA only and was not associated with polymorphic variation of normal DNA. It is important to note that the present technical development in sequencing has shown the great importance of small genomic variations of individuals, although the potential of these variations in cancer development has only started to emerge (30).

Recently, the question concerning the role of DNA Pol ε has become more interesting because the 256 kDa catalytic domains of Pol ε were not found to be essential for DNA replication, DNA repair or cell viability (31). In the present

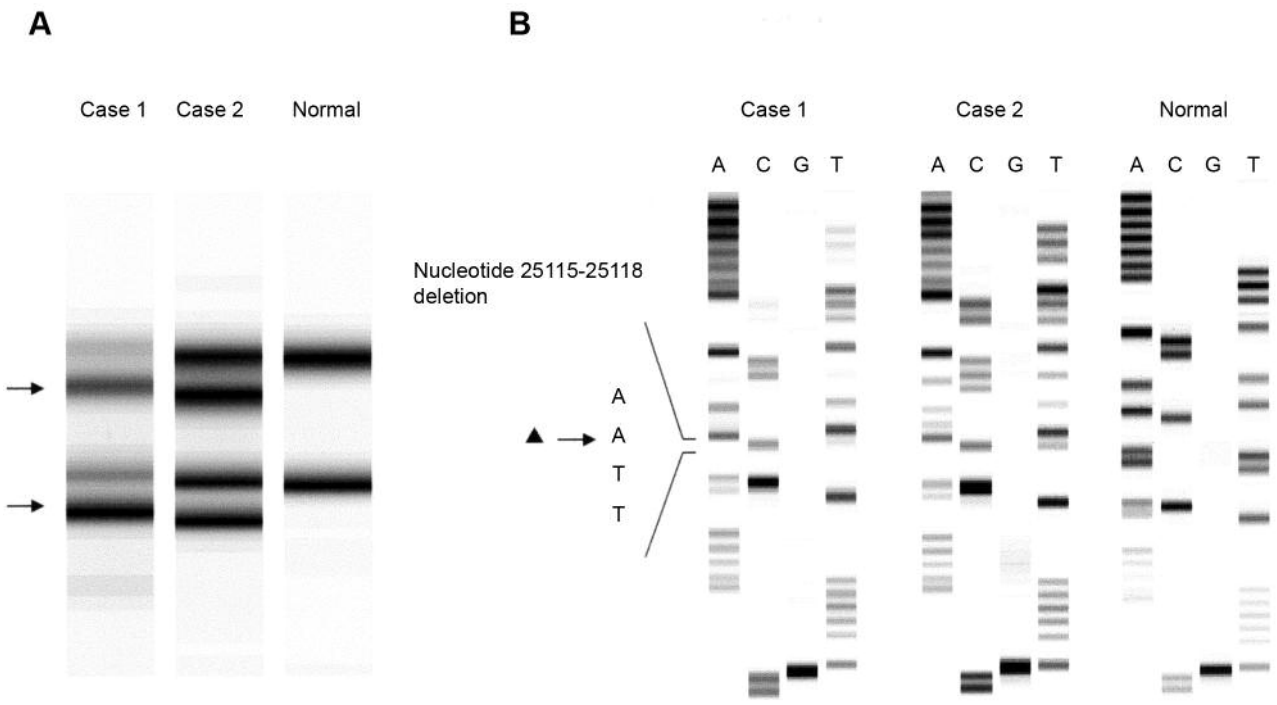

Figure 1. *(A) SSCP analysis of the PCR products derived from primer set 19. Abnormal patterns were observed in cases 1 and 2. (B) The DNA sequences of the PCR products of cases 1 and 2, and normal tissue sequence. Arrow, AATT deletion at nucleotide 25115-25118.*

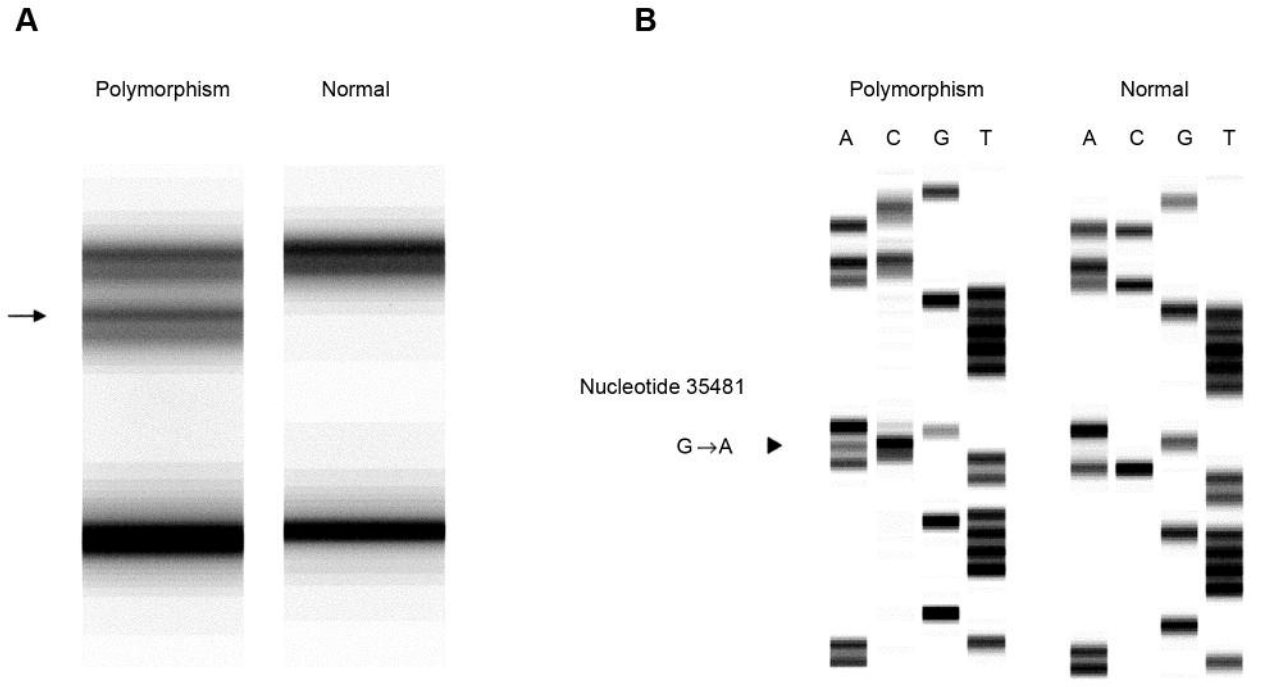

Figure 2. *(A) SSCP analysis of the PCR products derived from primer set 14. An abnormal pattern was observed. (B) The DNA sequence of the PCR product of the polymorphism, and normal tissue sequence. Arrow, G to A transition at nucleotide 35481.*

Table II. *Nucleotide sequence variation found in the 55 kDa small subunit of pol ε as compared with BAC clone data as reference (GenBank accession number AL591767) in 24 breast cancer samples (number 1-24) and 3 control samples from normal lymph nodes (numbers 25-27) and 1 control placental DNA (number 28).*

Sample number	Polymorphisms/mutations
1	g-a transition in intron 14, at nt. 35481
2	no change
3	aatt deletion in intron 18, at nt. 25115-25118 g-a transition in intron 14, at nt. 35481
4	no change
5	no change
6	g-a transition in intron 14, at nt. 35481
7	g-a transition in intron 14, at nt. 35481
8	g-a transition in intron 14, at nt. 35481
9	g-a transition in intron 14, at nt. 35481
10	no change
11	g-a transition in intron 14, at nt. 35481
12	no change
13	no change
14	g-a transition in intron 14, at nt. 35481
15	g-a transition in intron 14, at nt. 35481
16	no change
17	no change
18	no change
19	no change
20	aatt deletion in intron 18, at nt. 25115-25118
21	no change
22	no change
23	no change
24	g-a transition in intron 14, at nt. 35481
25	g-a transition in intron 14, at nt. 35481
26	g-a transition in intron 14, at nt. 35481
27	g-a transition in intron 14, at nt. 35481
28	no change

study, the Pol ε gene variations occurred in the B subunit of Pol ε in two out of the 24 cases of human breast cancer examined. Being present in the intron this mutation does not affect the protein code, but may influence splicing efficiency and expression levels. Previously we have found that mutations in the catalytic subunit of Pol ε were not detectable in breast cancer, at least in the highly conserved sequences covering 35% of reading frames (32).

A recent study has demonstrated that splice variants are common in Pol β, especially in bladder cancer. Examination of cDNA clones revealed that 49 out of 89 sequenced cDNA clones had large deletions, each starting or ending at exon-exon junctions. Because these deletions occurred at exon-exon junctions and were seen in cDNA but not in genomic DNA (13), the influence of Pol β in carcinogenesis could be expected to be routed through post-transcriptional regulation of mRNA. Against this background, further studies on pol ε could concentrate on expression levels, the presence and expression of splice variants (33) in cancer, and the variation in colocalization of splice variants with PCNA in various phases of the cell cycle (34).

The results presented in this study constitute the second report of potential Pol ε mutation in human tumors. We have now extended the search of the same type of deletion in colon cancer. Irrespective of the character of the change, mutational or less dramatically polymorphic in character, this genomic change still waits further proof of cancer association. Tumor development is often associated with multiple genetic alterations within a particular cell. The mutator phenotype, characterized by increased mutation rate and uncontrolled replication of cells, is hypothesized to be a common feature in many neoplasms (35). DNA pol ε can be associated with this pattern because changes in the gene, for example, could influence proofreading and repair of the damaged parts of the DNA strand, as shown by the fidelity association of the corresponding subunit in yeast (28).

Acknowledgements

This study was supported by the Turku University Hospital, Turku University Foundation, Cancer Foundation for South-West Finland and the Finnish Cancer Foundation.

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Received July 7, 2008
Accepted September 25, 2008