Original Article

CCAAT/Enhancer-Binding Protein α (*CEBPA*) Polymorphisms and Mutations in Healthy Individuals and in Patients with Peripheral Artery Disease, Ischaemic Heart Disease and Hyperlipidaemia

(*CEBPA* gene / C/EBPα protein / hyperlipidaemia / ischaemic heart disease / mutation / peripheral artery disease / polymorphism)

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Abstract. The CCAAT/enhancer-binding protein α, encoded by the intronless *CEBPA* gene, is a transcription factor that induces expression of genes involved in differentiation of granulocytes, monocytes, adipocytes and hepatocytes. Both mono- and bi-allelic *CEBPA* mutations were detected in acute myeloid leukaemia and myelodysplastic syndrome. In this study we also identified *CEBPA* mutations in healthy individuals and in patients with peripheral artery disease, ischaemic heart disease and hyperlipidaemia. We found 16 various deletions with the presence of two direct repeats in *CEBPA* by analysis of 431 individuals. Three most frequent repeats included in these deletions in *CEBPA* gene are CGCGAG (493-498_865-870), GG (486-487_885-886), and GCCAAGCAGC (508-517_907-916), all according to GenBank Accession No. NM_004364.2. In one case we identified that a father with ischaemic heart disease and his healthy son had two identical deletions (493_864del and 508_906del, both according to GenBank Accession No. NM_004364.2) in *CEBPA*. The occurrence of deletions between two repetitive sequences may be caused by recombination events in the repair process. A double-stranded cut in DNA may initiate these recombination events in adjacent DNA sequences. Four types of polymorphisms in the *CEBPA* gene were also detected in the screened individuals. Polymorphism in *CEBPA* gene 690 G>T according to GenBank Accession No. NM_004364.2 is the most frequent type in our analysis. Statistical analysis did not find significant differences in the frequency of polymorphisms in *CEBPA* in patients and in healthy individuals with the exception of P4 polymorphism (580_585dup according to GenBank Accesion No. NM_004364.2). P4 polymorphism was significantly increased in ischaemic heart disease patients.

Introduction

The CCAAT/enhancer-binding protein α (C/EBPα) is a member of the leucine zipper family of transcription factors that are required for the differentiation of myeloid cells, adipocytes, hepatocytes, and airway epithelial cells (Darlington et al., 1998; McKnight, 2001; Keeshan et al., 2003; Inoue et al., 2004; Martis et al., 2006; Friedman, 2007; Fuchs, 2007). Members of this family consist of N-terminal transactivation domains (TAD1 and TAD2), a basic region (DBD) able to interact with specific DNA sequences and a C-terminal leucine-rich dimerization region (ZIP) (Fig. 1). C/EBPα is encoded by an intronless gene that is 2783 bp long and maps to human chromosome 19q13.1 (Hendricks-Taylor et al., 1992). The full-length protein C/EBPα is a 42 kDa (C/EBPα-42) isoform, and initiation of translation from

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Abbreviations: AML – acute myeloid leukaemia, Cdk – cyclin-dependent kinase, *CEBPA* gene or C/EBPα protein – CCAAT/enhancer-binding protein α gene or protein, DBD – DNA-binding region, E2F – transcription factor involved in cell-cycle regulation and synthesis of DNA in mammalian cells, HDL – high-density lipoprotein, MDS – myelodysplastic syndrome, PAD – peripheral artery disease, SWI/SNF – SWItch/Sucrose NonFermentable, PCR – polymerase chain reaction, TAD – transactivation domain, ZIP – leucine zipper.

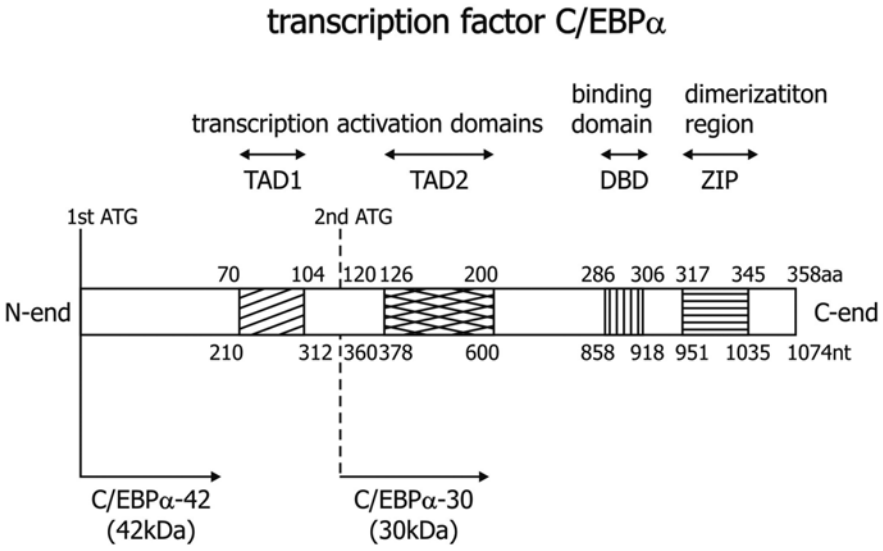

Fig. 1. The location of functional domains within the C/EBPα protein. Numbers directly above the schema indicate the amino acids of the human C/EBPα. Numbers directly under the schema indicate nucleotides (GenBank Accession No. NM_004364.2). The full-length 42 kDa form of C/EBPα protein and shorter 30 kDa form of this protein are also shown.

an internal initiation codon results in a truncated 30 kDa (C/EBPα-30) protein lacking TAD1 (Fig. 1). C/EBPα is a critical tumour suppressor in the haematopoetic system. *CEBPA* mutations (somatic or germ-line), which occur in acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS), are involved in abrogation of C/EBPα expression and/or function (Pabst et al., 2001; Gombart et al., 2002; Nerlov, 2004; Lin et al., 2005; Fuchs et al., 2008). Two types of *CEBPA* mutations are most frequent: frameshift mutations in the N-terminal region, and in-frame insertions or deletions in the C-terminal DBD and ZIP regions. Both these *CEBPA* mutation types can occur as mono- or bi-allelic mutations. Changes in the N-terminal region lead to expression of C/EBPα-30, which has a dominant-negative effect and altered DNA binding (Schwieger et al., 2004). Mutations in the C-terminal region prevent homodimerization or hetero-dimerization of C/EBPα, which is a prerequisite for DNA binding (Barjestah van Waalwijk van Doorn-Khosrovani et al., 2003). A relatively favourable prognosis of AML patients (event-free and overall survival) is associated with *CEBPA* mutations (mainly the double mutations, the combination of N- and C-terminal mutation types), and therefore these *CEBPA* mutations gained interest as a prognostic marker (Pabst et al., 2009; Wouters et al., 2009).

In addition to its transcriptional activity, C/EBPα inhibits cell proliferation without binding to DNA (Johnson, 2005; Schuster and Porse, 2006) through protein-protein interactions. These interactions include increasing *p21* gene expression and post-translational stabilization of p21 protein (Timchenko et al., 1996, 1997), disruption of E2F complexes (Porse et al., 2001), inhibition and degradation of cyclin-dependent kinase 2 (Cdk2) and Cdk4 (Wang et al., 2001, 2002) and interaction with the SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodelling complex (Pedersen et al., 2001; Műller et al., 2004).

In a previous study we have analysed the presence of polymorphisms and mutations in the *CEBPA* of patients with haematologic malignancies (Fuchs et al., 2008). During this study we have found an interesting class of six various deletions in the *CEBPA* gene, which involved a direct repeat of at least 2 bp. These mutations are characterized by the loss of one of two identical repeats at the ends of the deleted sequence. Now, we report the presence of these *CEBPA* deletions in healthy individuals and in patients with peripheral artery disease, ischaemic heart disease and hyperlipidaemia. We found 16 various deletions of this type in the *CEBPA* gene. We analysed changes in the *CEBPA* gene of patients with peripheral artery disease, ischaemic heart disease and hyperlipidaemia because two recent papers have shown that polymorphism of the *CEBPA* gene (Olofsson et al., 2008) and chemical mutagenesis of mice, which accidentally targeted the *CEBPA* gene (Juan et al., 2008), are connected with higher serum triglyceride levels and strong elevation of plasma high-density lipoprotein (HDL) cholesterol.

Material and Methods

Genomic DNA, RNA and cDNA preparation

After informed consent patient- or healthy individual-derived peripheral blood mononuclear cells (264 peripheral artery disease patients, 45 ischaemic heart disease patients, 24 hyperlipidaemia patients and 98 healthy individuals) were Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) purified and total RNA and genomic DNA were isolated. Complementary DNA was synthesized from total RNA using SuperScript

II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA).

Identification of CEBPA mutations

The polymerase chain reaction (PCR) was carried out using genomic DNA or cDNA and Advantage-GC Genomic Polymerase Mix (Clontech Laboratories, Inc., Mountain View, CA). Two or in some cases four overlapping primer pairs, which cover the entire coding region of human *CEBPA,* were used (Pabst et al., 2001). In some samples with abnormal sequencing results, an additional pair of PCR primers was exploited (BF, BR) (Liang et al., 2005). The positions of the primers complementary to the C/EBPα cDNA sequence (GenBank Accession No. U_34070) were FPP1 (562-585) 5'-TCGCCATGCCGGGAGAACTCTAAC-3', RPP1 (1114-1137) 5'-CTGGTAAGGGAAGAGGCCG-GCCAG-3', FPP2 (1060-1079) 5'-CCGCTGGTGAT-CAAGCAGGA-3', RPP2 (1739-1762) 5'-CACG-GCTCGGGCAAGCCTCGAGAT-3', F1 (563-582) 5'-CGCCATGCCGGGAGAACTCT-3', R1 (850-869) 5'-GCCTTGGCCTTCTCCTGCTG-3', F2 (829-848) 5'-GACCTGTTCCAGCACAGCCG-3', R2 (1122-1141) 5'-GCGGCTGGTAAGGGAAGAGG-3', F3 (1084-1103) 5'-CGCGAGGAGGATGAAGCCAA-3', R3 (1426-1450) 5'-CCCGGTACTCGTTGCTGT-TCTTGTC-3', F4 (1404-1423) 5'-GGGCAAG-GCCAAGAAGTCGG-3', R4 (1651-1670) 5'-CCT-CACGCGCAGTTGCCCAT-3', BF (816-835) 5'-CGAGTTCCTGGCCGACCTGT-3', BR (1119-1138) 5'-GCTGGTAAGGGAAGAGGCCG-3'.

PCR was performed in 25 μl reaction containing 40 mM Tris-HCl (pH 9.3 at 25 °C), 85 mM KOAc, 5% DMSO, 0.1% DMSO, 1.1 mM $Mg(OAc)_2$, 1 M GC-Melt (Clontech Laboratories, Inc.), 0.2 mM each of dATP, dTP, dGTP, and dTTP, 0.2 μM each of forward and reverse primer, 1.7 units of *TtH* DNA Polymerase from Advantage GC Genomic Polymerase Mix (Clontech Laboratories, Inc.) and 50 ng genomic DNA. Amplification was performed with an initial heat denaturation step for 4 min at 94 °C followed by 39 cycles of 30 s denaturation at 94 °C, 1 min of annealing at 65 °C (FPP1-RPP1 and F1-R1, 64 °C for FPP2-RPP2, 63 °C for F2-R2, 60 °C for F3-R3, F4-R4 and BF-BR) and 100 s of elongation at 72 °C in the Peltier Thermal Cycler (MJ Research, Inc., Watertown, MA). PCR products were electrophoresed on agarose gels, electroeluated from the pieces of gel, purified and sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) in both directions in ABI 3 100 DNA Genetic Analyzer (Applied Biosystems) or similarly using Genome Lab DTCS-Quick Start Kit and CEQ 8000 genetic analysis system (Beckman Coulter Inc., Fullerton, CA). GenBank Accession No. NM_004364.2 was used for evaluation of obtained sequences. Some earlier papers about *CEBPA* mutations used GenBank Accession No. U_34070, and therefore our results are described according to both GenBank Accessions No. NM_004364.2 and No. U_34070.

Results and Discussion

Patients with peripheral artery disease

In total 62.1 % of peripheral artery disease (PAD) patients analysed by us had abnormal levels of lipids and/or lipoproteins in the blood. Arterial hypertension was detected in 76.4 % of these patients, ischaemic heart disease in 40.4 % and type 2 diabetes mellitus in 43.5 % of the screened PAD patients.

Detection of polymorphisms

We detected four types of polymorphisms in *CEBPA* (Table 1). The most frequent type of polymorphism in *CEBPA* in the Czech Republic is P3 polymorphism 690 G>T according to GenBank Accesion No. NM_004364.2 and 1281 G>T according to GenBank Accesion No. U_34070 (Table 1). This type of polymorphism was detected in all groups of screened individuals and was present not only in heterozygous, but also in homozygous form. Statistical analysis did not find significant differences in the frequency of P3 polymorphism and of other polymorphisms in *CEBPA* in patients and in healthy individuals with the exception of P4 polymorphism (580_585dup according to GenBank Accesion No. NM_004364.2). P4 polymorphism was significantly increased in ischaemic heart disease patients (Table 1).

The rarest type of polymorphism is P1 (Table 1), which was described as silent mutation (Gombart et al.,

Table 1. Summary of patient samples examined for CEBPA polymorphisms and mutations

Screened individuals	No.	P1[a] hom.[e]	P1 het.[f]	P2[b] hom.	P2 het.	P3[c] hom.	P3 het.	P4[d] hom.	P4 het.	P3+P4 het.	Mutations het.
Peripheral artery disease	264	0	1	0	2	0	48	0	7	3	23
Ischaemic heart disease	45	0	0	0	0	0	8	1	3	0	5
Hyperlipidaemia	24	0	0	0	0	0	4	0	1	0	5
Healthy persons	98	0	1	0	1	3	23	0	4	0	6

[a] P1-polymorphism 402 G>A according to GenBank Accesion No. NM_004364.2 and 993 G>A according to GenBank Accesion No. U_34070
[b] P2-polymorphism 573 C>T according to GenBank Accesion No. NM_004364.2 and 1164 C>T according to GenBank Accesion No. U_34070
[c] P3-polymorphism 690 G>T according to GenBank Accesion No. NM_004364.2 and 1281 G>T according to GenBank Accesion No. U_34070
[d] P4-polymorphism 580_585dup according to GenBank Accesion No. NM_004364.2 and 1171_1176dup according to GenBank Accesion No. U_34070
[e] homozygous
[f] heterozygous

Table 2. Deletion types and their numbers in screened individuals[a]

Nucleotide change[b]	Nucleotide change[c]	Amino acid change[b,c]	Comment	Repetition (nucleotide position and sequence)	Frequency of mutations[d]
493_864del	1084_1455del	E166_R289del	Deletion in TAD2 and partial deletion of DBD	493-498 and 865-870 CGCGAG	25
486_884del	1077_1475del	E163_A295del	Deletion in TAD2 and partial deletion of DBD	486-487 and 885-886 GG	23
508_906del	1099_1497del	A177_K302del	Deletion in TAD2 and partial deletion of DBD	508-517 and 907-916 GCCAAGCAGC	20
502_900del	1093_1491del	D168_R300del	Deletion in TAD2 and partial deletion of DBD	502-503 and 901-902 GA	6
152_520del	743_1111del	L52_A174del	Deletion of TAD1 and partial deletion of TAD2	152-157 and 521-526 CGCTGG	3
480_836del	1071_1427del	K161_D279del	Deletion in TAD2	480-483 and 837-840 CAAG	3
260_411del	851_1002del	Q87fsX119	Frameshift and stop in TAD2, deletion of TAD1	254-259 and 406-411 GCCGGC	1
61_430del	652_1021del	P23fsX36	Frameshift and stop in TAD2, deletion of TAD1	61-66 and 431-436 AGCCCC	1
508_819del	1099_1410del	A170_K273del	Deletion of TAD2	508-513 and 820-825 GCCAAG	1
666_1083del	1257_1674del	H224fsX282	Deletion of DBD and ZIP	666-668 and 1084-1086 CGG	1
30_402del	621_993del	E10fsX35	Deletion of TAD1 , frameshift and STOP in TAD2	30-33 and 403-406 GCCG	1
547_555del	1138_1146del	P182_P184del	Deletion in TAD2	547-545 and 546-551 CCGCCG	1
527_854del	1118_1445del	A176fsX208	Deletion in TAD2,frameshift and stop before DBD	527-530 and 855-858 CCGG	1
497_904del	1088_1495del	E167_K302del	Deletion in TAD2 and partial deletion of DBD	497-498 and 905-906 AG	1
558_617del	1149_12098el	P187_L206del	Partial deletion of TAD2 (P rich region)	558-559 and 618-619 GC	1
489_888del	1080_1479del	P164_V296del	Deletion in TAD2 and partial deletion of DBD	489-490 and 888-889 GC	1

[a] 431 screened individuals (264 peripheral artery disease patients, 45 ischaemic heart disease patients, 24 hyperlipidaemia patients and 98 healthy individuals)
[b] GenBank Accession No. NM_004364.2
[c] GenBank Accession No. U_34070
[d] Frequency of mutations = number of individuals carrying one to three deletions or more deletions of these types

2002). Polymorphism P4 (Table 1) was first reported as a mutation (Fröhling et al., 2004; Lin et al., 2005). Lin et al. (2005) detected this mutation in seven (39 %) of 19 healthy volunteers and in 20 (20 %) of the AML patients. AML patients remained positive for this mutation at complete remission and the authors considered this mutation as an insignificant change for leukaemogenesis. It has been demonstrated that this in-frame insertion of six nucleotides in the second transactivation domain (TAD2) of *CEBPA* represents a germ-line polymorphism (P194_H195dup) in a proline-histidine-rich region of C/EBPα (Resende et al., 2007). This type of polymorphism was also shown in healthy individuals (Resende et al., 2007; Wouters et al., 2007). We confirmed these results and we have found four healthy persons (4.1 %) heterozygous for P4 polymorphism in 98 screened healthy persons (Table 1). This P4 polymorphism is the most frequent type in Thailand (Leecharendkeat et al., 2008). Valk et al. (2004) described in a study of 285 AML cases that *CEBPA* mutations are present predominantly in two distinct gene expression clusters, but in-frame insertion of six nucleotides (P4 polymorphism) did not belong to these gene expression clusters. *In vitro* studies (Wang et al., 2001) have suggested that this proline-histidine rich region of C/EBPα may play a role in the regulation of proliferation through the inhibition of two key cyclin-dependent kinases (Cdk2 and Cdk4) that drive cell-cycle progression, but *in vivo* experiments did not support this observation (Porse et al., 2006).

Detection of mutations

We detected 16 various deletions with the presence of two direct repeats in *CEBPA* (Table 2) in 43 individuals out of 431 screened individuals (264 peripheral artery disease patients, 45 ischaemic heart disease patients, 24 hyperlipidemia patients and 98 healthy individuals). Some of the screened individuals carried two, three or more various deletions of this type (Table 3). These mutations are characterized by the loss of one of two identical repeats at the ends of the deleted sequence. Three most frequent repeats included in these deletions in *CEBPA* gene are CGCGAG (493-498_865-870), GCCAAGCAGC (508-517_907-916) and GG (486-487_885-886), all according to GenBank Accession No. NM_004364.2.

In Fig. 2 an example of these deletions is shown (patient No. 177A with peripheral artery disease). This deletion 152_520del, 152-157 and 521-526 CGCTGG repetition according to GenBank Accession No. NM_004364.2 (Table 2), causes deletion of TAD1 and partial deletion of TAD2 and loss of the transactivating potential of mutated C/EBPα in the transcription of target genes involved in granulocyte differentiation. The ability of this mutant to arrest cell proliferation will possibly be lost through diminished E2F binding, as suggested by structural analysis.

In one case we identified that a father with ischaemic heart disease and his healthy son had two identical dele-

Table 3. Number of individuals with or without deletions with repetitions

Diagnosis	Number of screened individuals	Number of individuals carrying one deletion	Number of individuals carrying two deletions	Number of individuals carrying three deletions	Number of individuals carrying more than three deletions
Peripheral artery disease	264	5	8	6	4
Ischaemic heart disease	45	1	2	0	2
Hyperlipidaemia	24	3	2	1	1
Healthy individuals	98	1	0	3	1

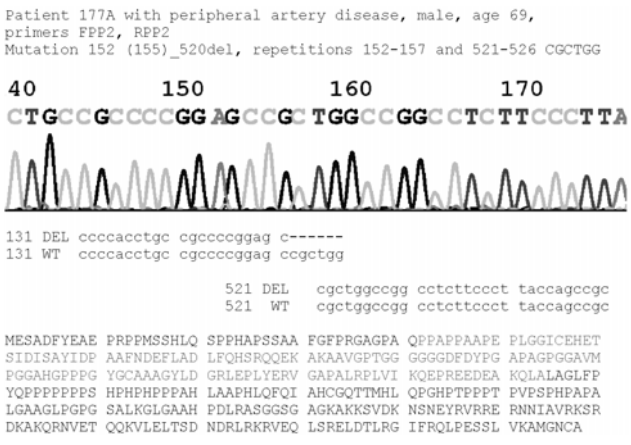

Fig. 2. An example of detection of deletion in *CEBPA*, which involves a direct repeat. Sequencing chromatogram of patient No. 177A with peripheral artery disease indicating the presence of deletion at cDNA nucleotide position 152 (155)_520del, 152-157 and 521-526 CGCTGG repetition according to GenBank Accession No. NM_004364.2, causing deletion of TAD1 and partial deletion of TAD2 and loss the of transactivating potential of mutated C/EBPα in the transcription of target genes involved in granulocyte differentiation. The same mutation was found in another patient with peripheral artery disease (165A, female, age 65) and in a healthy individual (100209).

tions (493_864del and 508_906del, both according to GenBank Accession No. NM_004364.2) in *CEBPA*.

We detected deletions with the presence of two direct repeats in *CEBPA* not only in peripheral artery disease patients, ischaemic heart disease patients and hyperlipidemia patients, but also in five of 98 healthy individuals (Table 3). Therefore, it is possible that intronless *CEBPA* may be more susceptible to genotoxic stress than other genes containing introns.

The occurrence of deletions between two repetitive sequences may be caused by recombination events in the repair process, for example an unequal crossover. A double-stranded break in DNA can initiate these recombination events in adjacent DNA sequences (Szostak et al., 1983; Fishman-Lobell et al., 1992). Rearrangements between repetitive sequence elements are the cause of genomic instability in both prokaryotes and eukaryotes. A large subset of mutations that inactivate genes are deletion events between two short regions of sequence homology in bacteria, yeast and in humans (Thacker et al., 1992; Bzymek and Lovett, 2001). Direct repeats of between 2 bp and 10 bp were found in the immediate vicinity of many deletions analysed. Direct repeats are a feature of a number of recombination, replication or repair-based models of deletion mutagenesis. They are mutation "hot spots". In humans, deletion or duplication between repeated DNA sequences contribute to human genetic diseases, both of nuclear genes and in the mitochondrial genome (Krawczak and Cooper, 1991; Lestienne and Bataillé, 1994; Abeysinghe et al., 2003; Chuzanova et al., 2003; Samuels et al., 2004; Krishnan et al., 2008).

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