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Missense Polymorphism (C/T224) in the Human Cathepsin D Pro-fragment Determined by Polymerase Chain Reaction—Single Strand Conformational Polymorphism Analysis and Possible Consequences in Cancer Cells

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Overexpression of cathepsin D in human breast cancers is associated with a higher risk of relapse and metastasis. Also, pro-enzyme routing is altered in several tumoral mammary cell lines, leading to its hypersecretion. MCF7 cells compared to normal kidney carry a C → T transition at position 224 in the cathepsin D gene which converts Ala to valine in its pro-fragment. Using polymerase chain reaction-single strand conformational polymorphism analysis (PCR-SSCP), the variant T allele frequency was found to be 23–30%, and equally distributed in cancer and normal cells. Six to nine per cent of genotypes were homozygous T/T, 34–41% were heterozygous T/C and 50–59% were homozygous C/C. Moreover, genotypes were identical in 19 out of 20 matched sets of tumoral mammary cells and normal white blood cells from the same patients. Loss of heterozygosity was noted in 1 case. C/T224 transition is thus not due to a somatic event. However, this missense polymorphism might modify pro-cathepsin D secretion and/or maturation in breast cancer cells.

Key words: polymorphism, breast cancer, cathepsin D, PCR-SSCP
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INTRODUCTION

THE ROLE of tissue proteases in facilitating cancer metastasis and invasion has been proposed. One of the major mechanisms suggested involves degradation of the basement membrane following secretion of pro-enzymes and their activation in the extracellular medium [1]. Cathepsin D (cath-D), an acidic lysosomal protease, is overexpressed in most breast cancer cells, and hypersecreted by some breast cancer cell lines [2]. Moreover, this protease can be induced by oestrogens and growth factors [3], and its elevated concentration in breast cancer cytosols is associated with increased risk of metastasis [4–6]. The cath-D gene is located on chromosome 11, and cloning and sequencing of cath-D cDNA from MCF7 breast cancer cells has shown five base pair changes [7] as compared to the human kidney cath-D cDNA sequence [8]. Four of these changes are silent mutations and one (C → T at position 224) converts the seventh alanine, which is located in the cath-D pro-fragment, to valine and increases its hydrophobicity. Pro-enzyme activation requires removal of the pro-fragment [9], which might also be involved in routing of pro-cath-D to lysosomes via an alternative membrane pathway not mediated by mannose-6-phosphate receptors [10].

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In addition, Capony and colleagues [11] have shown that maturation of pro-cath-D is delayed, and its secretion substantially increased in several breast cancer cell lines as compared to normal mammary cells from reduction mammoplasties or to the ZR75-1 cell line, from which cloned cath-D-cDNA was previously not found to be mutated at position 224 [12].

These findings raised two major questions concerning the significance of the C → T mutation: (i) is it a somatic mutation found only in cancer cells, or a polymorphism? (ii) Whatever the mechanism, does the resulting amino acid change in the pro-fragment modify routing and/or activation of the pro-enzyme?

To address the first question, we adapted a combination of polymerase chain reaction (PCR) and single strand conformational polymorphism (SSCP) [13] to reliably and rapidly screen single point mutations at position 224, and defined the C → T conversion frequency in 22 cancer cell lines, 32 breast cancer tissues and 29 non-malignant cells. We then analysed cath-D secretion and maturation in 10 cell lines using pulse chase experiments.

MATERIALS AND METHODS

Biological materials

We analysed the following human cell lines—from breast cancer: MCF7, BT20, T47D,MDA-MB231 (described previously in [14]), ZR75-1 [15], BT474 [16], EFM19 [17] and MDA-MB436 (obtained from Dr Pasqualini, Paris, France); from non-malignant epithelial mammary tissue: HMT 3522

[18], MCF-10 [19], BKST18 (obtained from M. Stampfer, Berkeley, U.S.A.) and HBL100. We also studied BG1 ovarian cells [20], cervical Hela cells, endometrial Ishikawa cancer cells (obtained from Dr Kuramoto, Kanajawa, Japan), Hep G2 liver cancer cells (obtained from Dr Corvol, Paris, France) and, from skin, melanoma cancer cell lines MM96 (obtained from Dr Gill, Adelaide, Australia) and M1477 (obtained from Sanofi, Montpellier, France), A431 (described previously in [14]) and fibroblasts (obtained from Dr Von Figura, Göttingen, Germany).

Normal and tumoral breast biopsies were collected by surgery for diagnostic and therapeutic purposes. All tissue samples were frozen in liquid nitrogen immediately after surgery and stored at −80°C in our Department of Cell Biology. When necessary, tissue cells were subjected to an intermediate extraction step for cath-D immunoassay as described previously [11], and the resulting cell pellets were collected for DNA analysis.

Whole blood samples were collected for other diagnostic or prognostic purposes. They were centrifuged (10 min, 1500 *g*) and the white blood cell (WBC) fraction was kept at −80°C until use. We followed the ethical criteria for clinical research (Helsinki, 1964).

DNA extraction

Frozen biopsies were sectioned using a Reichert-Jung Frigocut cryostat: a first 5-μm thick section was collected for a histological control, then the next three to four (20-μm thick) were saved for PCR analysis. Genomic DNAs from cell pellets, WBC and tissue sections were extracted for PCR amplification, as described previously [21].

PCR conditions

The following primers were used to amplify a sequence of convenient size for SSCP analysis:

Upstream primer: 5′ CCG CTG CAC AAG TTC ACG 3′
Downstream primer: 5′ CTT GAG CAC CTC GGG AAT 3′

The resulting 144 bp PCR fragment spanned from +124 to +267 in exon 2 and contained most of the pro-cath-D sequence (Fig. 1) [22]. We used Taq DNA polmerase and buffer from Promega (Madison, Wisconsin, U.S.A.) and the Perkin Elmer Thermal Cycler (Cetus, U.S.A.). Samples were cycled 35 times at 95°C for 20 s, 63°C for 20 s and 72°C for 40 s. When PCRs were performed for sequencing, an upstream/downstream primer ratio of 1/12 was used and 15 cycles were added.

SSCP procedures

Unless otherwise specified in the figure legends, we routinely used SSCP conditions which we optimised. PCR products were first run on a 5% polyacrylamide minigel (Eurogentec, Belgium), the amplified 144 bp DNA was then excised with a razor, followed by elution in 50 μl water for 1 h at 65°C. The purified PCR products (7–10 μl) were mixed with 1% SDS–100 mM EDTA, then 7 μl of stop solution (USB, U.S.A.) was added. Samples were denatured at 95°C for 3 min, rapidly cooled on ice, then loaded on to a 15% polyacrylamide gel containing 5% glycerol. In parallel, unheated aliquots and DNA molecular weight markers were loaded during preliminary experiments to monitor migration of the double-stranded (ds) DNAs remaining in the heated samples. SSCP gels were run in 0.5xTBE (Tris 0.9 M, borate 0.9 M, EDTA 25 mM) buffer at room temperature for 6 h under increasing voltage (50 mV, 100 mV, 150 mV), and then silver stained using the silver stain kit (Biorad, Paris, France). All experiments were performed in duplicate.

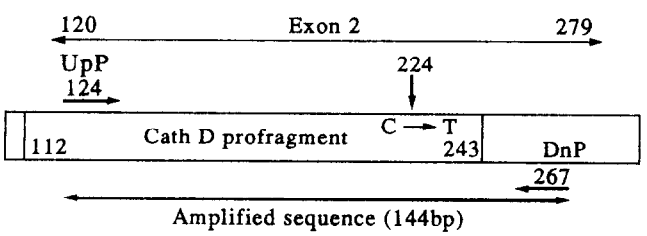

Fig. 1. Schematic representation of the amplified cath-D sequence. A 144 bp fragment was selected for PCR amplification within exon 2 of the cath-D gene. The upstream (UpP) and the downstream (DnP) primers are located in the sequence coding for the pro-fragment and for the short chain of the mature enzyme, respectively.

Other methods

Sequencing was performed using the sequenase 2 kit (USB). Cath-D immunoassays and pulse chase experiments were carried out as described previously [11].

RESULTS

Adaptation of the PCR-SSCP analysis conditions to detect heterozygosity in the C224 → T transition

We used the SSCP analysis technique to discriminate between two pro-cath-D sequences which only differ by a single base substitution at position 224. The two breast cancer cell lines from which the cDNA sequence was known, MCF7 (T224) [7] and ZR75-1 (C224) [12], were used to monitor the technique. Another cell line of unknown genotype (T47D) was also analysed. Since the original [13] and variant [23] SSCP protocols were at first unsucessful (not shown), we varied several technical parameters as described in the Materials and Methods. Under these conditions (Fig. 2), SSCPs only discriminated the upper strand since with the lower strand either C or T runs with a very similar mobility in all cell lines. The upper strand (called the variant strand) exhibited one different conformer in MCF7 and T47D cells whereas, unexpectedly, both conformers were found in ZR75-1 cells. We consequently deduced that the MCF7 variant strand corresponded to the mutated (T) sequence, the T47D cells variant strand to the normal (C) sequence and we suspected ZR75-1 to be heterozygous. This interpretation of our SSCPs was confirmed by direct sequencing of products generated by an asymmetric PCR (Fig. 2). MCF7 cell lines carry 2 to 5 chromosome 11s [24] which might originate from successive duplications of one chromosome, the other one probably being lost [25]. We thus could not discriminate between T/T homozygosity and T/- hemizygosity. The above SSCP conditions allowed us: (i) to define optimal conditions for large scale PCR/SSCP analysis of the mutation at position 224 in patients and (ii) to classify the 144 bp sequences into three apparent allelotype groups, normal C/C224 homozygotes, C/T224 heterozygotes and variant T/T224 homozygotes.

The C224 → T transition corresponds to a missense polymorphism and not to a somatic mutation in cancer cells

To determine the frequency and distribution of the T224 allele, we performed our PCR/SSCP protocol on 22 cell lines, 32 mammary tumours and 29 non-cancer tissues. As summarised in Tables 1 and 2, the three allelotypes were similarly distributed in all cells, regardless of their origin, the normal C/C224 group being prevalent. We then compared the SSCP patterns of DNAs purified from breast cancer tissues and the white blood cells of

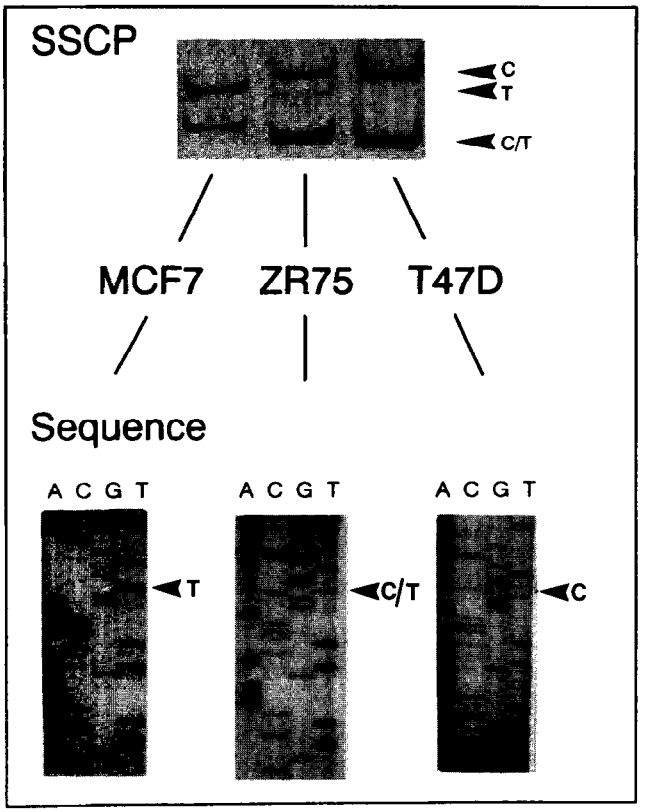

Fig. 2. Analysis of cath-D pro-fragment genotype at position 224. Genomic DNAs from three cell lines: MCF7, ZR75-1 and T47D were amplified by PCR and the resulting 144 bp fragments were subjected to SSCP analysis or sequenced. Top: SSCP conditions were as described in Materials and Methods. Migration of the remaining ds DNAs are not shown. Bottom: direct sequencing was carried out using the upstream primer. Genotype at position 224 is arrowed.

the same patients (only 20 of 32 were available), to investigate a possible somatic mutation. In 19 out of 20 cases, identical allelotypes were found in WBC and tumoral biopsies (Fig. 3). However, for 1 patient the tumour DNA was homozygous C/C224, whereas the WBC DNA was heterozygous. This discrepancy most likely corresponded to a loss of heterozygosity on chromosome 11p15, as frequently occurs in breast cancer cells [26]. Our results indicate that variants at position 224 in the pro-cath-D sequence are missense polymorphisms, strongly suggesting that the MCF7 genotype is not caused by a somatic mutation.

Table 1. Analysis of cathepsin D genotypes at position 224 in 22 cell lines

	T/T*	C/T	C/C*
Breast cancers	MCF7 or derived	EFM19	BT474†
		MDA-MB 231	MDA-MB435
		ZR75-1	MDA-MB436
			BT20,T47D
Normal breast		BKST18	HMT3522
		HBL100	MCH10
Others	MM96	A431, M1447	BG1, Hela
		Fibro, CD45	Ishikawa
			HepG2

Genotypes were determined by SSCP analysis as in Fig. 2. *Hemizygous (T/− or C/−) cannot be excluded. The allelic balance of heterozygotes was not quantified. †A monosomy 11 was described for the BT474 cell line [15], which is therefore likely to be C/−.

Table 2. Distribution of the T allele in several cell types

	Presence of T allele No. chromosome 11		No. of cases	Genotypes T/T	T/C	C/C
Breast cancer*	(64)	23%	(32)	6%	34%	59%
Normal tissue‡	(58)	26%	(29)	7%	38%	55%
Cell lines§	(44)	30%	(22)	9%	41%	50%

Genotypes were determined by SSCP analysis as in Fig. 2 .*Hemizygous (T/− or C/−) cannot be excluded. The allelic balance of heterozygotes was not quanitifed. †A monosomy 11 was described for the BT474 cell line [15] which is therefore likely to be C/−. ‡Eight reduction mammoplasties, 15 WBC, three cutaneous biopsies and three placenta samples. §22 cell lines detailed in Table 1.

Possible consequence of C224 → T polymorphism on pro-cath-D routing to lysosomes

We reported previously that cath-D overexpression, routing and processing are altered in several mammary cancer cell lines leading to excess secretion of the pro-enzyme [11]. The mechanism of this excess secretion is not known, and may be due to altered balance between the different transport mechanisms involved and the enzyme to be transported [9, 10, 11, 27]. We also considered the possibility that the increased pro-enzyme secretion may be facilitated by the Ala to Val change in the pro-fragment. We, therefore, investigated several cell lines to determine whether there was a correlation between the enzyme genotype at position 224 and its processing pathway, as studied by pulse chase experiments. Figure 4 shows that presence of the T224 allele tended to co-segregate with high percentages of pro-cath-D secretion and decreased maturation of the enzyme into its 34 kDa lysosomal form. However, the low number of cell lines studied did not allow statistical comparisons. We, therefore, cannot draw any conclusion on the role of this polymorphism in the increased secretion of pro-cath-D, nor can we exclude this possibility.

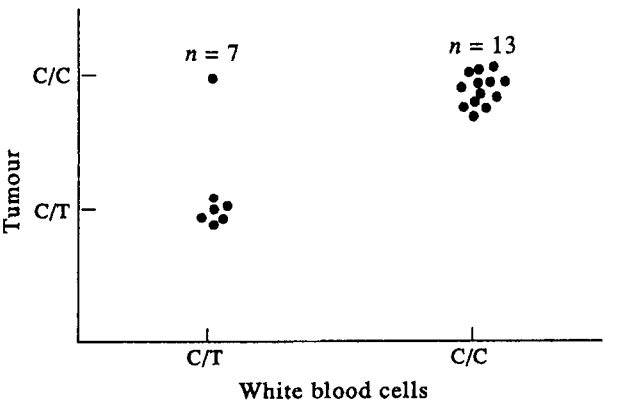

Fig. 3. Comparison of genotypes at position 224 in tumour and white blood cells from the same patients. DNAs from 20 matched sets of tumoral and normal cells were prepared and SSCPs were performed as described in the Material and Methods. No patients of the T/T224 genotype were found in this group.

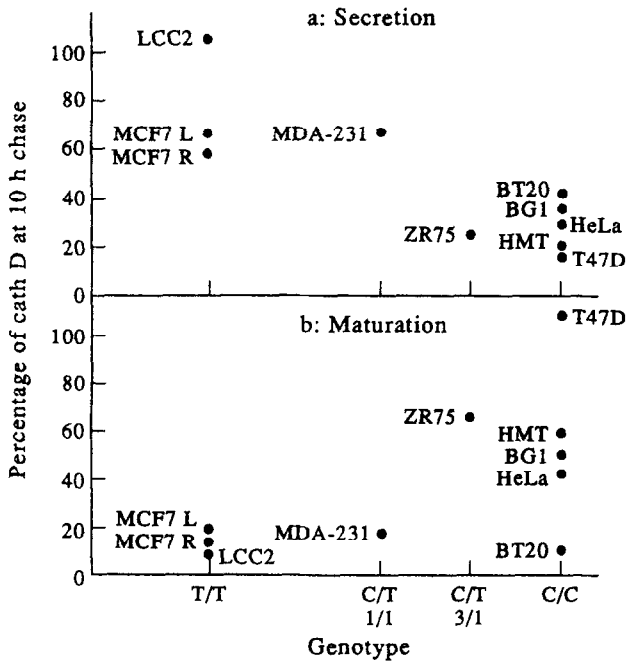

Fig. 4. Routing of the pro-cath-D enzyme as a function of its corresponding genotype. Cancer cells of mammary origin were labelled for 1 h with [^{35}S]methionine and chased for 10 h as described previously in Capony and colleagues [11]. Secretion of the immature 52 kDa form (a) and maturation of the enzyme into its processed 34 kDa form (b) are represented as a percentage of remaining cath-D after 10 h of chase. MCF7, MDA-MB231 and BT20 values are those described in Capony and colleagues [11]. LCC2 values are unpublished observations from P. Coopman.

Moreover, there was no correlation between the presence of the T224 allele and the extent of cath-D gene expression, irrespective of the cell origins. For instance, cellular cath-D varied from 50 to 800 fmoles/μg DNA in several MCF7 sublines displaying the same T/T224 genotype.

DISCUSSION

We have demonstrated, by SSCP analysis of PCR-amplified genomic fragments, that in MCF7 cells the C → T mutation at position + 224 in the cath-D gene, which converts the second Ala to Val in the enzyme pro-fragment, is due to polymorphism, and not to a cancer-specific somatic mutation. This C → T224 polymorphism provides an additional tool for genetic linkage analysis in the 11p15 chromosome region, where the cath-D gene is normally located [7], and which is often deleted in cancer cells [26]. The T/T224 genotype is rare. Only 2 cases out of 29 were found in WBC of non-cancer patients.

In breast cancer cells, cath-D is characterised by increased gene expression and increased pro-enzyme secretion. Cath-D gene overexpression is correlated with metastasis, both *in vitro* and *in vivo*, but as shown here, it is not correlated with the Ala → Val polymorphism. Increased pro-enzyme secretion, originally described in several breast cancer cell lines, was also observed *in vivo* in pleural effusions [28]. It is frequently associated with and is possibly due to increased gene expression and saturation of mannose-6-phosphate receptors [29]. However, the role of this increased secretion of pro-cath-D in metastasis is not yet clear. By contrast, it seems that maturation of the pro-enzyme is required to facilitate metastasis [30, 31]. Though maturation of the secreted pro-cath-D has not been observed in cell lines grown in buffered medium, *in vivo* the secreted pro-cath-D could be maturated and act on degrading basement membrane. In this case, the Ala → Val polymorphism, by increasing hydrophobicity of the pro-fragment, might alter the routing and/or the maturation of the pro-enzyme.

We have shown that pro-enzyme secretion was increased and its intracellular maturation altered in most cell lines carrying the T224 allele (MCF7 and variants and MDA-MB231). More work is required concerning possible involvement of C/T224 polymorphism in modulating pro-enzyme activation under *in vitro* and *in vivo* conditions.

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Phase II Trial of Anaxirone (TGU) in Advanced Colorectal Cancer: an EORTC Early Clinical Trials Group (ECTG) Study

Eduard E. Holdener, Michel Clavel *, Cristina Sessa, Wim ten Bokkel Huinink, Pierre Siegenthaler, Christian Ludwig, Otto Klepp, Georgette Renard, Genevieve Decoster and Herbert M. Pinedo

Anaxirone, a rationally synthesised triepoxide derivative, was given to 46 patients with metastatic colorectal cancer. Good risk patients received 800 mg/m^2 as a rapid intravenous injection every 4 weeks, whereas poor risk patients received 650 mg/m^2. Of 46 patients, 45 were evaulable for toxicity and 42 for efficacy analysis. There were 37/45 patients with poor risk, showing no difference in toxicity as compared to good risk patients. The major toxic effect was myelosuppression with 34% of all patients experiencing grade 3 or 4 leucopenia; thrombocytopenia was less frequent. Locoregional phlebitis occurred in 66% of the patients. There was no objective tumour response to anaxirone in 42 evaluable patients. Only 4 patients achieved stabilisation of the disease lasting maximally up to 248 days. Anaxirone is inactive in metastatic colorectal cancer.
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INTRODUCTION

ANAXIRONE (α-β-TRIGLYCIDYLURAZOL, TGU, NSC-332488) is a rationally synthetised triepoxide derivative which has shown high antitumour activity in five animal tumour models [1]. In clinical phase I investigations, the dose-limiting toxic effect was myelosuppression. Dose-related gastrointestinal toxicity and dose-unrelated mild to moderate phlebitis were also seen [2–4]. This study was undertaken to determine if responses can be achieved with anaxirone in advanced colorectal cancer, and to further characterise the toxic effects of anaxirone in this patient population.

PATIENTS AND METHODS

Eligibility criteria consisted of measurable recurrent or metastatic colorectal disease not amenable to curative surgery and/or radiotherapy, life expectancy of at least 3 months, a WHO-Zubrod ECOG performance status of grade ≤ 2, age ≤ 75 years, pretreatment white blood cell (WBC) count of $\geq 4.0 \times 10^9$/μl, platelet count $\geq 100 \times 10^9$/μl, serum creatinine < 132 μmol/l and bilirubin < 25.6 μmol/l, no prior radiotherapy or chemotherapy within 4 weeks (6 weeks for nitrosoureas or mitomycin) and no brain involvement or leptomeningeal disease. The protocol has been submitted and