Oncogene (2004) 23, 2236–2240

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Intragenic deletion of *CDH1* as the inactivating mechanism of the wild-type allele in an HDGC tumour

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Mutations in *CDH1*, encoding E-cadherin, are the underlying genetic defect in approximately one-third of the hereditary diffuse gastric cancer (HDGC) families described so far. Tumours arising in these families show abnormal or absence of E-cadherin expression, following the model of tumour suppressor gene inactivation. A single study has been reported showing inactivation of the *CDH1* wild-type allele in tumour cells from HDGC families either by promoter methylation or by somatic mutation. In order to find the genetic alteration responsible for the presence of diffuse gastric cancers in four members of a Caucasian family, we have screened the coding sequence of *CDH1* for germline mutations and searched for the second inactivating hit in the tumour samples. In this family, we have found a germline splice-site mutation in all members affected by gastric cancer and, in one tumour, a somatic deletion affecting at least exon 8 of *CDH1*. Our results show that a *CDH1* intragenic deletion is the second hit inactivating the wild-type allele, in one of the tumours in this family.

Oncogene (2004) **23,** 2236–2240. doi:10.1038/sj.onc.1207335
Published online 8 December 2003

Keywords: E-cadherin; hereditary diffuse gastric cancer; HDGC; mutation; intragenic deletion; second hit

In 1999, the syndrome of hereditary diffuse gastric cancer (HDGC) was defined by the International Gastric Cancer Linkage Consortium (IGCLC) (Caldas *et al*., 1999) as any family that fulfils one of the following criteria: (1) two or more documented cases of diffuse gastric cancer in first/second-degree relatives, with at least one diagnosed before the age of 50 years; or (2) three or more cases of documented diffuse gastric cancer in first/second-degree relatives, independent of age. Knowledge regarding the genetic basis of HDGC has greatly increased in the last few years, due to the discovery of germline mutations in the gene coding for E-cadherin (*CDH1*) within families with this cancer predisposing syndrome (Gayther *et al*., 1998; Guilford P *et al*., 1998; Guilford PJ *et al*., 1999; Iida *et al*. 1999; Keller *et al*., 1999; Richards *et al*., 1999; Shinmura *et al*., 1999; Yoon *et al*., 1999; Avizienyte *et al*., 2001; Dussaulx-Garin *et al*., 2001; Humar *et al*., 2002; Jonsson *et al*., 2002; Oliveira *et al*., 2002; Yabuta *et al*., 2002; Wang *et al*., 2003).

Members of HDGC families have a high predisposition to develop diffuse carcinomas of the stomach and an elevated risk for other types of cancer, like lobular carcinoma of the breast (Caldas *et al*., 1999). Most *CDH1* germline mutations found in HDGC are truncating and evenly distributed along the gene (Oliveira *et al*., 2003). In the cases of HDGC reported so far, which harbour germline *CDH1* mutations, the inactivation of the wild-type allele is due to hypermethylation of the *CDH1* gene promoter or somatic mutations (Grady *et al*., 2000).

We screened for *CDH1* inactivation in a newly identified HDGC family. Germline mutations of *CDH1* were screened in the affected members with diffuse gastric cancer of this family. The second somatic hit in the E-cadherin locus was also investigated.

This Caucasian family fulfilled the criteria for HDGC (cancers in all affected members were classified as diffuse/isolated cell-type carcinoma), and the mutation screening in all affected members revealed the presence of a novel *CDH1* germline heterozygous splice-site mutation (1135^IVS8 + 5del8ins5) at the splice donor-site of intron 8 (Figure 1a and b). This alteration is predicted to alter splicing of the *CDH1* gene. In fact, RT–polymerase chain reaction (PCR) and cloning studies, performed in normal gastric mucosa from Subject II-1, revealed the coexistence of the wild-type transcript and three distinct aberrant transcripts (for details see Figure 2). The most common aberrant transcript is predicted to give rise to a truncated protein product, without both the transmembrane and cytoplasmic domain, thus being expected to be functionally inactive. The second transcript results in an in-frame deletion that is predicted to produce a smaller

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[6]We wish it to be known that, in our opinion, the first two authors should be regarded as joint first authors
Received 30 July 2003; revised 3 November 2003; accepted 11 November 2003

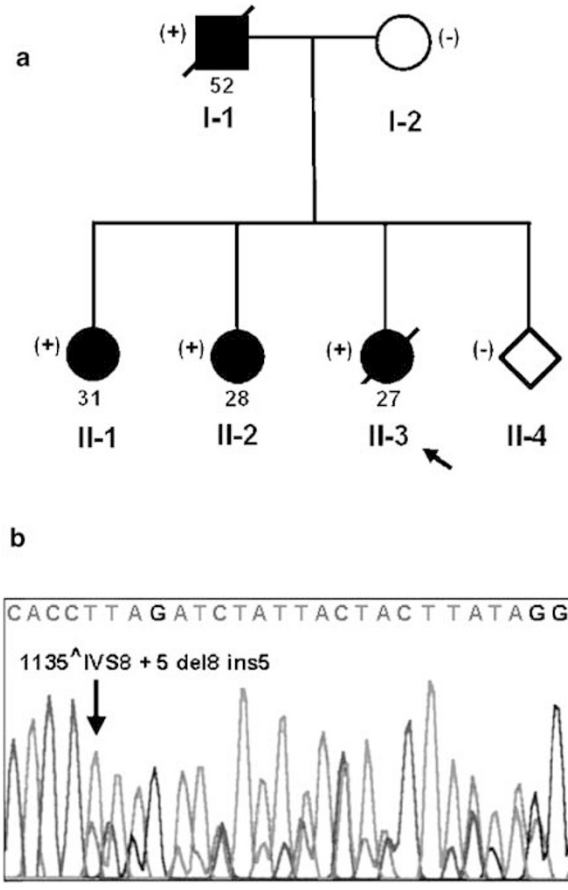


Figure 1 Family subjects, biological samples and mutation analysis. (**a**) Pedigree from a Caucasian HDGC family of European origin. The study protocol was reviewed and approved by the appropriate Ethics Committees and blood samples, paraffin blocks and the family history was obtained with informed consent. The proband in this family (subject II-3 – arrow), a female with signet-ring cell gastric carcinoma diagnosed by endoscopic biopsies, underwent a total gastrectomy at the age of 27 years. The proband's father (subject I-1) was diagnosed with a metastatic gastric cancer at the age of 52 years and total gastrectomy revealed advanced-stage signet-ring cell gastric carcinoma (T3N2Mx). The proband's two older sisters (Subjects II-1, II-2) were referred to a medical geneticist and integrated in a programme of genetic and endoscopic screening, in spite of being asymptomatic. These two sisters were diagnosed with early diffuse gastric cancer by multiple endoscopic biopsies performed before they underwent total gastrectomy. The gastric cancer lesions found in both stomachs were signet-ring cell carcinomas with pTNM staging T2N0M0 and T1N0M0 in Subjects II-1 and II-2, respectively. In both patients, the tumour was multifocal. Peripheral blood was available from Subjects I-2, II-1, II-2, II-3 and II-4, paraffin-embedded normal and tumour tissues from Subject I-1, II-1, II-2 and II-3, and frozen tissue from normal gastric mucosa from subject II-1. The age of onset of the tumours is shown underneath the symbols; solid symbols, diffuse gastric cancer histologically confirmed; (+), carriers of the *CDH1* germline mutation; (−), subjects who did not carry the mutated *CDH1* allele in the germline DNA; arrow, proband. (**b**) Genomic DNA was isolated from white blood cells using standard methods and from paraffin-embedded tumour or normal mucosa by phenol/chloroform extraction, using standard methods, after microdissection of areas where tumour cells occupied more than 90% of the tissue section. One tumour sample was analysed per patient. All 16 coding regions, intron–exon boundaries and the promoter region of *CDH1* were amplified by PCR from germline and somatic (one tumour) DNA of each family member. Primer sequences, PCR conditions, SSCP/heteroduplex analysis and sequencing analysis were based on those reported previously (Oliveira *et al*., 2002)

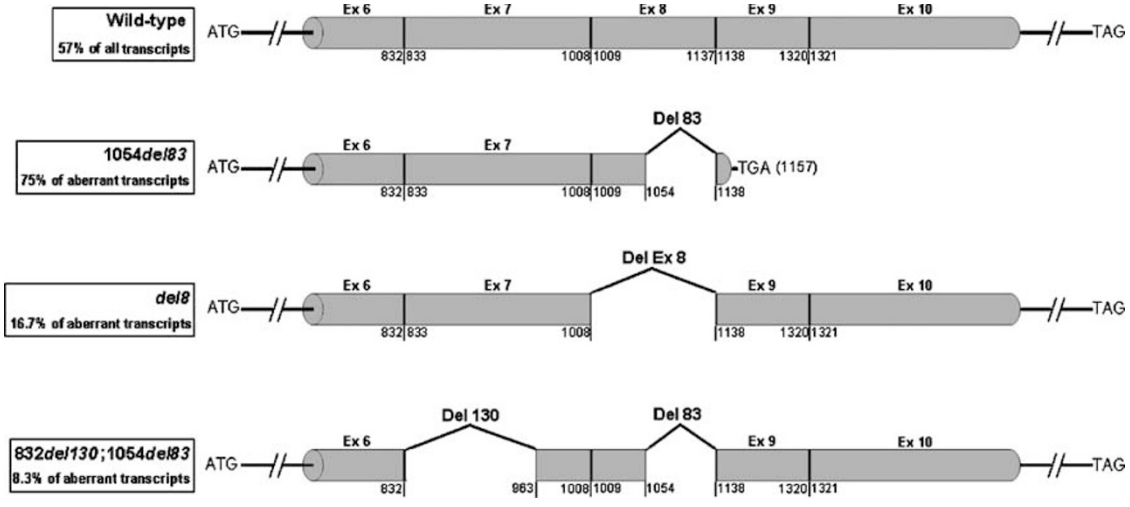


Figure 2 Scheme of the RNA transcripts produced by the *CDH1* germline mutation found in subject II-1. RNA was isolated from frozen normal gastric mucosa from the subject II-1 using Trizol Reagent (Life Technologies, Inc., Paisley, Scotland) following the manufacture's instructions. Total RNA was used to synthesize first-strand cDNA with SuperScript II (Life Technologies, Inc., Paisley, Scotland). *CDH1* transcripts were amplified using a primer set designed to amplify exons 6–10 (E-cad 6F1: 5′-TGA GGA TCC AAT GGA GAT TTT-3′; E-cad 10R1: 5′-GAC CTC AAA AGG TAC CAC ATT CGT CAC-3′). RT–PCR products were cloned using the ZeroBlunt TOPO PCR Cloning Kit (Invitrogen, Groningen, The Netherlands). Colony-PCR was performed using M13 universal primers, and the products were sequenced to determine the exact structure of 28 transcripts, 16 (57%) of them being wild-type transcripts. From the remaining 12 (43%) aberrant transcripts, the most common (9/12–75%) had an 83 bp out-of-frame deletion inside exon 8, as a consequence of cryptic splicing at position 1054 resulting in a downstream stop codon, at position 1157 in exon 9. The second transcript (2/12–16.7%) had skipping of the complete sequence of exon 8, resulting in an in-frame deletion, lacking only the amino acids encoded by exon 8. A rare transcript (1/12–8.3%) had two out-of-frame deletions, the first was a 130 bp deletion inside exon 7, as a consequence of cryptic splicing at position 963, and the second was 83 bp, as a consequence of the cryptic splicing at position 1054; altogether, these two deletions give rise to an in-frame deletion of 213 bp, resulting in an E-cadherin protein lacking 71 amino acids

E-cadherin protein, lacking only the amino acids encoded by exon 8. The third transcript was rare and results from two out-of-frame deletions, leading to an in-frame deletion, thus giving rise to a smaller E-cadherin protein, lacking 71 amino acids encoded by part of exon 7 and part of exon 8 (Figure 2).

We searched for the inactivation of the wild-type allele in a tumour sample from each individual of the family. Initially, promoter methylation analysis was performed in Subjects I-1 and II-1, and no methylated alleles were found (Figure 3a, Table 1). In Subjects II-2 and II-3, the methylation status of the *CDH1* promoter region could not be evaluated due to the small amount of tumour DNA.

Subsequently, LOH analysis was performed in a tumour sample from each individual of the family. No loss of genetic material was found in any of the tumours using *CDH1* distal and proximal microsatellite markers (Figure 3b, Table 1). Further, the screening for somatic mutations in the entire coding sequence of the *CDH1* gene did not reveal deleterious *CDH1* somatic mutations in all tumour samples analysed. The only sequence variants found in tumour samples were two common polymorphisms in the promoter region (−160 C/A) and exon 13 (2076 C/T) that revealed to be heterozygous in all cases, except for Subject I-1 that was constitutionally homozygous for both polymorphisms. In tumour sample from Subject II-1, we were unable to amplify the wild-type sequence of exon 8, whereas the common polymorphisms −160 C/A and 2076 C/T were still present heterozygously (Figure 3c, Table 1). These results reflect the presence of a somatic intragenic deletion of at least exon 8 in tumour DNA. To confirm this hypothesis and exclude a duplication of the mutant exon 8, we have developed a semiquantitative PCR-based technique. We analysed, in subject II-1, the

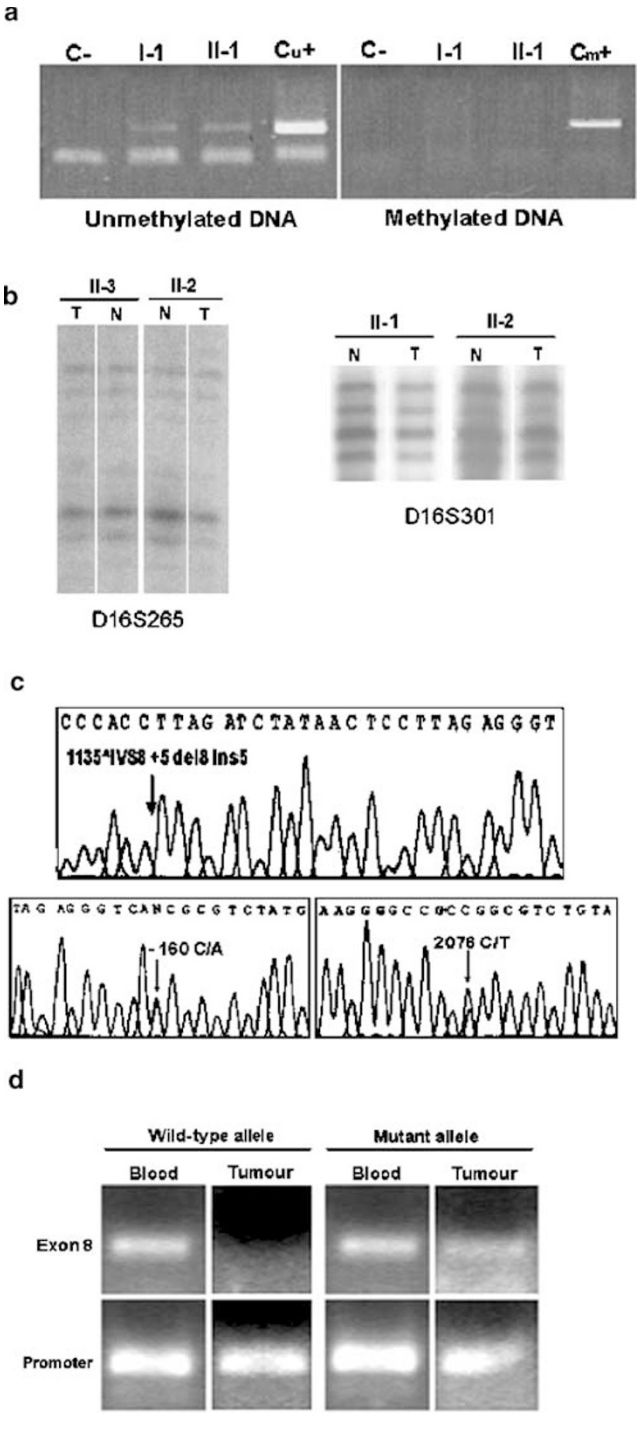
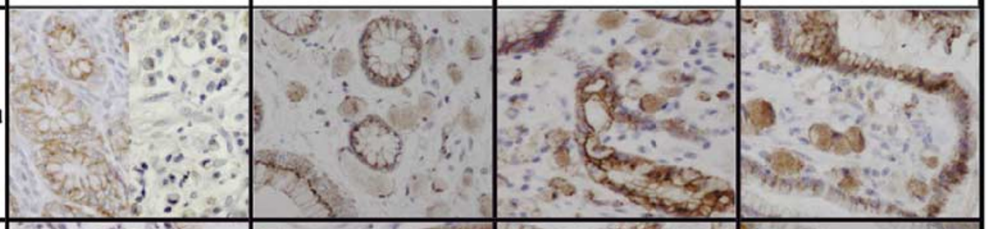
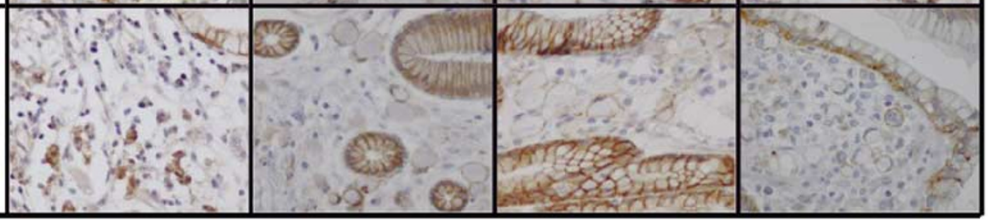


Figure 3 Methylation, LOH, sequencing and multiplex PCR analysis of tumours from the family subjects. (**a**) Methylation analysis of the *CDH1* promoter region showing the presence of unmethylated alleles in the tumour DNA. Promoter methylation analysis was performed in microdissected tumour material from Subjects I-1 and II-1. *CDH1* promoter methylation analysis was performed using methylation-specific PCR (MS-PCR) and primers described by Graff *et al*. (1997) for CpG island 3 of the *CDH1* promoter. This assay entails initial modification of DNA by sodium bisulphite, converting all unmethylated, but not methylated, cytosines to uracil, and subsequent amplification with primers specific for methylated versus unmethylated DNA. C−, negative control (blank); Cu+, bisulphite-treated blood donor DNA; Cm+, bisulphite-treated blood donor DNA *in vitro* methylated with M.*Sss*I DNA MeTase. (**b,c**) LOH analysis showing retention of heterozygosity in tumour samples. Two microsatellite markers (D16S265 and D16S301) and two intragenic single-nucleotide polymorphisms (SNP −160C/A and 2076C/T) were used for LOH analysis. LOH analysis was performed using two different chromosome 16 microsatellite markers, D16S265 (distal to *CDH1*) and D16S301 (proximal to *CDH1*). The PCR products from tumour versus constitutional DNA, from all family members, were labelled by [α-^{32}P]dCTP during the amplification reaction. *CDH1* common polymorphisms (the promoter −160C/A transvertion and a silent substitution 2076C/T at exon 13) were used as intragenic markers for LOH analysis in tumour DNA versus constitutional DNA from all the family members, by SSCP/sequence analysis. Sequencing analysis of exon 8, promoter region and exon 13 of *CDH1* in the tumour sample from subject II-1 showing only the mutant sequence of exon 8 and the two alleles in the promoter and exon 13. Methods for somatic mutation analysis of *CDH1* are described in the legend of Figure 1. (**d**) Multiplex PCR analysis showing intragenic deletion of *CDH1* affecting exon 8 in Subject II-1. A multiplex PCR analysis was designed using primers flanking promoter −160C/A variant and a set of primers amplifying exon 8, where the mutation lay. The forward primer for *CDH1* exon 8 was combined with specific reverse primers for the wild-type or for the mutant exon 8 *CDH1* sequence in different PCR reactions (wild-type sequence: 5′-TCT AAG GAG TTA TAG ATC TAA G-3′; mutant sequence: 5′-TCT AAG GAG TTA TAG AAT TAC C-3′). We compared the intensity of the PCR product of exon 8 with the intensity of the promoter −160C/A using a Multianalysis GEL DOC 1000 (BIO-RAD, USA)

Table 1 Summary of the genetic and epigenetic changes of *CDH1* in tumour samples and patterns of immunoexpression of E-cadherin and β-catenin (× 40)

Family case	I-1	II-1	II-2	II-3
Promoter methylation	No methylation	No methylation	ND	ND
Somatic mutation	No mutation	Intragenic deletion encompassing at least exon 8	No mutation	No mutation
LOH status	No LOH	No LOH	No LOH	No LOH
E-cadherin expression				
β-catenin expression				

HECD-1 (R&D, Minneapolis, USA) and anti-β-catenin (BD Transduction Laboratories, Bedford, USA) monoclonal antibodies were used for the IHC expression study of E-cadherin and β-catenin according to the methods described previously (Machado *et al*., 1998; Nabais *et al*., 2003). ND, not determined

tumour DNA versus the blood DNA using primers flanking the heterozygous promoter and specific primers for the wild-type and the mutant exon 8 sequences in a multiplex PCR reaction. In tumour DNA from subject II-1, no wild-type sequence of exon 8 was present, the mutant exon 8 sequence was represented once and the heterozygous promoter was represented twice. In blood DNA, the mutant exon 8 as well as the wild-type exon 8 were represented once, and the heterozygous promoter region was represented twice, as expected. The result of this experiment showed that an intragenic deletion of the wild-type allele was occurring without a concomitant duplication of the mutant exon 8 (Figure 3d). Owing to the lack of tumour material, we were unable to map the exact minimal region of this deletion.

We also analysed the immunohistochemical expression of E-Cadherin in tissue sections containing normal and tumour mucosa (Table 1). Neoplastic cells displayed, in every case, aberrant E-cadherin expression, whereas adjacent nontumour mucosa exhibited normal lateral membrane expression. In neoplastic cells, E-cadherin was either absent or expressed in the cytoplasm and occasionally at the cell membrane (dotted pattern). Most probably, the cytoplasmic expression is due to the temporary accumulation of truncated protein in the endoplasmic reticulum (cytoplasm) on its way to degradation (Chen *et al*., 1999; Huber *et al*., 2001). The dotted pattern of E-cadherin expression observed in the membrane of some signet-ring cells might be due to the presence, at the cell membrane, of the rare translation products, derived from the germline mutation, that miss only part of the extracellular domain and that keep the transmembrane and cytoplasmic domains, although lacking the adhesion capabilities. Alternatively, in the tumour from Subject II-1, this dotted pattern may be derived from the somatically mutated allele. This observation has already been described for similar mutants lacking exon 8 in sporadic gastric cancer cells. As shown by Handschuh *et al*. (1999), this mutant E-cadherin still localized to the lateral regions of cell-to-cell contact sites, but caused multiple morphological and functional disorders and induced scattered morphology and invasive behaviour.

In summary, we were able to identify the mechanism inactivating the *CDH1* wild-type allele in the tumour sample from family member II-1. No mechanisms were

identified to explain the aberrant/absent E-cadherin expression in tumour samples from other family members, namely patient I-1, in which promoter hypermethylation, LOH and somatic mutations were excluded.

Since the majority of tumour cells from the members of this family showed aberrant expression of E-cadherin, we have searched to which extent downregulation of E-cadherin expression could affect other components of the adhesion complex, using immunohistochemical analysis.

Catenins (α- and β-catenin) showed aberrant patterns of expression in neoplastic cells in all members of the family (see Table 1 for β-catenin). In every case, we observed an abnormal pattern of expression of both α- and β-catenin (decreased or absent membranous expression and/or cytoplasmic immunoreactivity). These findings might be explained by the disruption of the adhesion complex by the absence of normal E-cadherin protein.

Finally, we analysed the cell differentiation pattern of the carcinoma cells by the immunohistochemical staining of mucins (MUC1, MUC2, MUC5AC and MUC6) and the trefoil peptide TFF1. We observed that neoplastic lesions retained regions of gastric differentiation (expression of MUC1, MUC5AC and TFF1) (data not shown), in keeping with what has been described in sporadic diffuse/isolated cell-type carcinomas by Machado *et al*. (2000).

In conclusion, this is the first report demonstrating that intragenic deletions within the *CDH1* gene can be responsible for the inactivation of the wild-type allele. This observation highlights the need for developing experimental protocols to identify, in the setting of HDGC families, the presence of germline or somatic intragenic deletions in *CDH1*, which are easily missed by mutation detection methods based on PCR of genomic DNA.

Acknowledgements
This study was funded by grants from Fundação para a Ciência e a Tecnologia, Portugal (Project: POCTI/35374/CBO/2000 and POCTI/CBO/40820/2001).

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