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KILLER/DR5 is a DNA damage–inducible p53-regulated death receptor gene

P53 (also known as *TP53*), the cellular 'gatekeeper for growth and division'[1], is the most commonly mutated gene in human cancer, and its inactivation contributes not only to tumour progression but also to resistance of cancer cells to chemotherapy[2,3]. p53 normally controls cell proliferation by causing cell-cycle arrest and apoptosis. Cell-cycle arrest is believed to be mediated by transcriptional activation of *CDKN1A* (also known as $p21^{WAF1/CIP1}$), an inhibitor of cyclin-dependent kinases[4]. p53 regulates expression of genes engaged in apoptosis, including *BAX* and *Fas/APO1*, but neither is required for *p53*-dependent apoptosis[5,6].

To explore the pathway of p53-dependent cell death, we carried out subtractive hybridization screening of a library enriched for doxorubicin-induced transcripts derived from chemosensitive PA-1 ovarian-teratocarcinoma cells[7]. Of 50,000 clones screened, six appeared to be induced at 10 hours after doxorubicin treatment of PA-1 cells (data not shown). Isolation of *CDKN1A* indicated that the subtractive screen had yielded at least one expected target[4]. *CTSD*, the gene encoding the Cathepsin-D protease that contributes to cytokine-induced apoptosis, was also isolated[8]. Another clone that appeared to be strongly induced by doxorubicin was further characterized. Database analysis revealed that this new gene (Fig. 1*a*) is a member of the *TNFR* family[9]. Based on the presence of a cytoplasmic death domain (Fig. 1*b*), induction of its expression by cytotoxic chemotherapy (Figs 2*a*,*b*), and induction of apoptosis by its overexpression (Fig. 2*f*), the new gene was named *KILLER/DR5*.

The human *KILLER/DR5* gene encodes a 411-amino-acid polypeptide (Fig. 1*a*) of predicted molecular weight of 45 kD. The protein contains a cytoplasmic C-terminal death domain, a transmembrane domain and a cysteine-rich extracellular N-terminal domain characteristic of the TNF-receptor family[9]. The closest homologue of KILLER/DR5 is DR4 (ref. 10), with 55% identity over a 454-amino-acid alignment (not shown); it also has homology with other TNF-receptor family members (Fig. 1*b*). KILLER/DR5 shares 64% identity with DR4, 28% with DR3, 19% with Fas/APO1, 29% with TNFR1 and 31% with

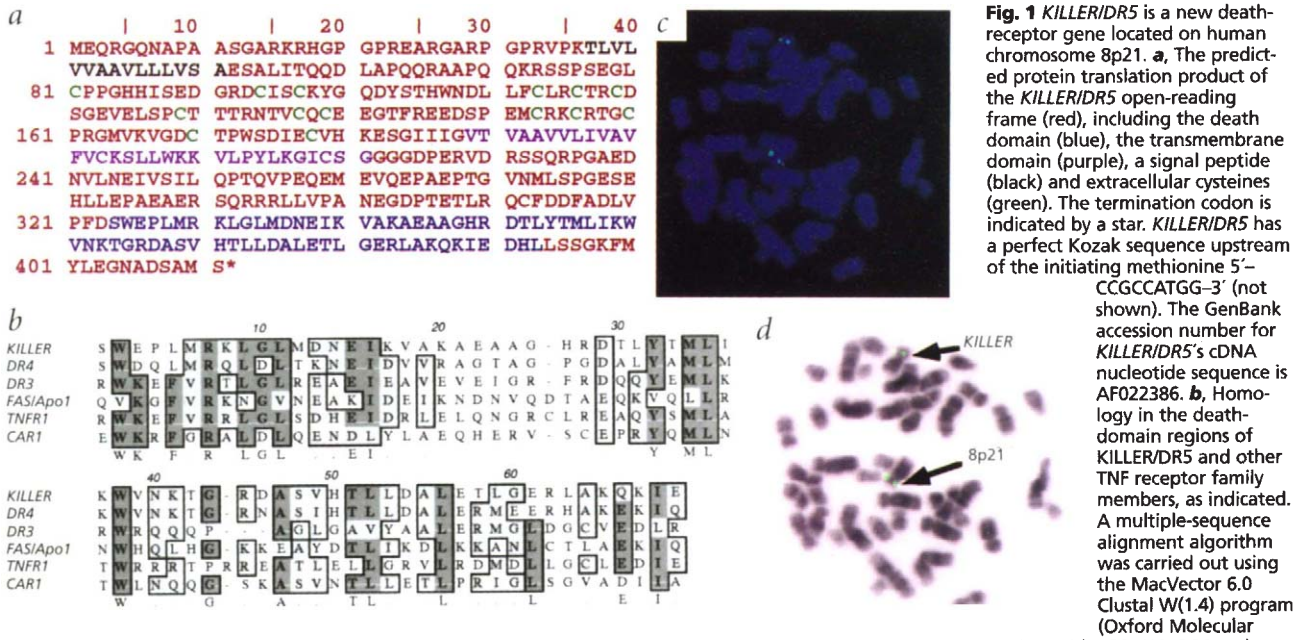

Fig. 1 *KILLER/DR5* is a new death-receptor gene located on human chromosome 8p21. ***a***, The predicted protein translation product of the *KILLER/DR5* open-reading frame (red), including the death domain (blue), the transmembrane domain (purple), a signal peptide (black) and extracellular cysteines (green). The termination codon is indicated by a star. *KILLER/DR5* has a perfect Kozak sequence upstream of the initiating methionine 5′–CCGCCATGG–3′ (not shown). The GenBank accession number for *KILLER/DR5*'s cDNA nucleotide sequence is AF022386. ***b***, Homology in the death-domain regions of KILLER/DR5 and other TNF receptor family members, as indicated. A multiple-sequence alignment algorithm was carried out using the MacVector 6.0 Clustal W(1.4) program (Oxford Molecular Group). The following amino acids were included within each death domain: KILLER/DR5 amino acids 324–390; DR4 amino acids 356–422, DR3 amino acids 346–410, Fas/APO1 amino acids 228–293, TNFR1 amino acid 330–398 and CAR1 amino acids 269–333. A predicted consensus is shown below the alignment. ***c***,***d***, Chromosomal localization of the human *KILLER/DR5* gene. Fluorescence *in situ* hybridization of a biotin-labelled P1 clone containing *KILLER/DR5* to a metaphase spread from a normal individual and DAPI counterstain (*c*). Computer-converted G-band–like image demonstrates that the hybridization is to chromosome 8, band p21 (*d*).

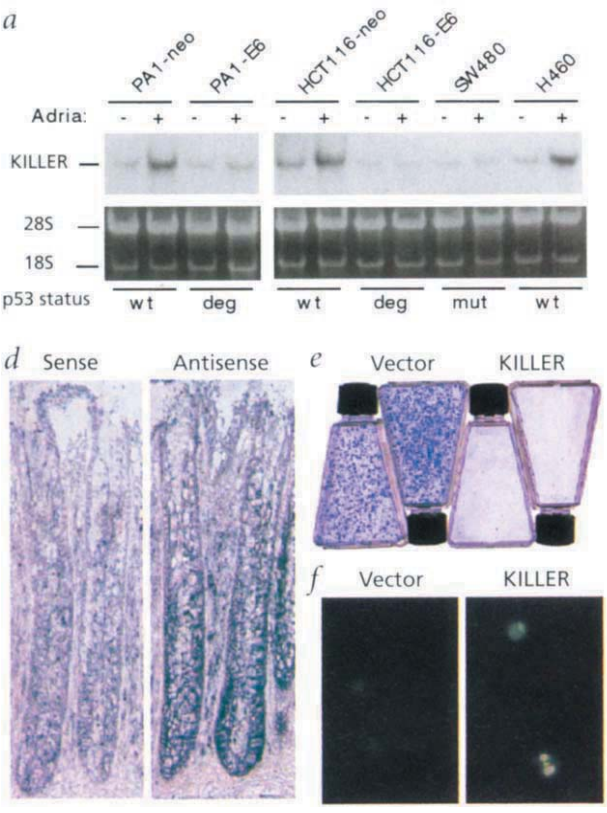

Fig. 2 *KILLER/DR5* is a p53-regulated growth-inhibitory and apoptosis-inducing gene. **a**, Northern analysis of *KILLER/DR5* expression (upper panels) in human cancer cell lines (as indicated) either untreated (–) or ten hours after continuous treatment (+) with an apoptosis-inducing concentration (0.3 μg/ml) of doxorubicin (Adria, Adriamycin). **b**, *KILLER/DR5* mRNA expression is increased in H460 lung cancer cells ten hours after continuous treatment with 0.3 μg/ml doxorubicin, 5 μM etoposide or a dose of 2,000 rads ionizing radiation at time zero. **c**, Induction of *KILLER/DR5* mRNA expression in mutant-p53–expressing colon, ovarian or breast cancer cells twelve hours after infection by either Ad-LacZ or Ad-p53. An ethidium stain of the RNA (lower panels) indicates equivalent RNA loading (10 μg) of the lanes in each experiment, and the *p53* status of each cell line is shown below the ethidium stains (*a*,*c*). **d**, Expression of *KILLER/DR5* in normal human colonic crypt epithelium. *In situ KILLER/DR5* mRNA was detected after hybridization to a digoxigenin-labelled antisense–*KILLER/DR5* RNA probe (right) or a sense–*KILLER/DR5* control RNA probe (left). **e**, KILLER/DR5 is a potent suppressor of colony growth after transfection of SW480 human colon-cancer cells. SW480 cells were transfected with pCEP4 vector (two left flasks) or pCEP4–*KILLER/ DR5* (two right flasks), selected with 0.25 mg/ml Hygromycin for two weeks and stained with Coomassie blue. Similar observations were made in HepG2 hepatoblastoma cells (not shown). **f**, Apoptosis induction after transient transfection of SW480 cells by pCEP4 vector (left) or pCEP4– *KILLER/DR5* (right). Of 100 cells counted, the percentage of TUNEL-positive cells was 9 and 32 for vector and *KILLER/DR5*, respectively. Expression of the cytoplasmic death domain was sufficient to induce apoptosis in SW480 cells and was toxic as a GST fusion when overexpressed in *E. coli* in the context of a GST fusion protein(data not shown).

CAR1. Northern analysis of the tissue expression of *KILLER/DR5* revealed a single transcript of 4.4 kb (not shown). Its expression was higher in tissues with rapid turnover than in nondividing tissues (data not shown). *KILLER/DR5* was localized to human chromosome 8p21 (Fig. 1*c*,*d*), a frequent site of chromosomal translocations[11] where tumour suppressors are believed to reside. We found no mutations in the death domain in eight colon-cancer cell lines with microsatellite instability.

Because *KILLER/DR5* was isolated as a doxorubicin-induced transcript, and because doxorubicin is a potent inducer of *p53* and p53-dependent growth suppression[2], we explored the possibility that *KILLER/DR5* is a p53-regulated gene. We examined *KILLER/DR5* mRNA expression in eleven human cancer cell lines of known p53 status (Fig. 2*a*–*c* and data not shown). *KILLER/DR5* expression was induced after doxorubicin exposure only if wild-type (wt) *p53* was present but not in cells in which it was mutated, degraded or not expressed. These results show strong correlation between *p53* status and induction of *KILLER/DR5* expression. *KILLER/DR5* expression was also inducible by other DNA-damaging agents, such as etoposide or ionizing radiation (Fig. 2*b*). To more directly test the hypothesis that *KILLER/DR5* is a p53-regulated gene, we infected human cancer cell lines lacking wild-type *p53* by using a wild-type p53-expressing adenovirus. *KILLER/DR5* expression was increased by wild-type *p53* (Fig. 2*c*). These results suggest that *KILLER/DR5* may be a mediator in *p53*-dependent apoptosis, even in the absence of DNA damage.

We investigated the *in situ* mRNA expression of *KILLER/DR5* to determine whether it correlates with apoptosis in human colon (Fig. 2*d*). We found that *KILLER/DR5* is expressed in the proliferating compartment of colonic crypt epithelia. This compartment has been observed to undergo DNA damage–induced apoptosis[12]. We believe that the lower basal *KILLER/DR5* mRNA expression in the nonproliferating colonic epithelial compartment may contribute to its relative resistance to DNA damage–induced apoptosis. We found that *KILLER/DR5* is a potent inhibitor of cancer-cell growth in colon (Fig. 2*e*) and liver (data not shown), as well as a potent inducer of cancer-cell apoptosis (Fig. 2*f*).

While this manuscript was in review, two groups using a DNA structure–based search strategy reported the independent identification of DR5, a death domain–containing DR4-homologous receptor for TRAIL[13,14]. In addition, an antagonist 'decoy' receptor for TRAIL, lacking an intracellular death domain (*TRID* or *DcR1* [refs 13,14]), was found to be expressed in many normal tissues but lost in many cancer cells. The decoy receptor effectively protected cells from TRAIL-induced death[13,14]. It was suggested that loss of the decoy receptor makes certain cancer cells more susceptible to TRAIL-mediated apoptosis.

In summary, we have isolated a new cell-death receptor gene whose expression is induced by DNA-damaging agents in a p53-dependent manner. *KILLER/DR5* is inducible by wild-type *p53* overexpression in the absence of DNA damage, and its overexpression leads to apoptotic death of cancer cells. Future experiments will focus on the role of *KILLER/DR5* in the development of cancer and chemoresistance, the mechanism of regulation by p53, the mechanism of apoptosis and whether *KILLER/DR5* is required for p53-dependent apoptosis. Members of the TNF receptor family signal apoptosis through adaptor molecules that directly activate the caspase cascade[9]. Our results suggest that TNFR-related proteins participate in DNA damage–induced cell death through p53-dependent regulation. Finally, the identification of *KILLER/DR5* provides a potential mechanism by which DNA damaging agents can lead to p53-dependent apoptosis; it further suggests strategies for cancer therapy, including *KILLER/DR5* gene replacement and screening for agents that upregulate its expression independent of p53.

Note in proof: The nomenclature of the *KILLER/DR5* gene is currently being considered by the HUGO nomenclature commitee.

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PTEN1 is frequently mutated in primary endometrial carcinomas

Endometrial cancer is the most common cancer of the female genital tract in America[1] and Japan[2]. However, our knowledge regarding the molecular mechanisms underlying endometrial carcinogenesis is limited. Although mutations of *p53* (also known as *TP53*; ref. 3) and *ras*[4] have been reported in endometrial cancers, the prevalence of alterations in these two genes is low.

The *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) gene, a candidate tumour-suppressor gene, was recently identified at chromosome 10q23.3 (refs 5,6). Studies thus far have demonstrated alterations of *PTEN* in Cowden disease[7], as well as in human brain, breast, prostate and kidney cancer cells[5,6]. In addition, a high rate of loss of heterozygosity (LOH) has been reported at chromosome 10q23–q26 in endometrial cancers[8]. To determine the possible involvement of *PTEN* in endometrial and other malignancies, we investigated a series of endometrial, colorectal, gastric and pancreatic carcinomas for intragenic sequence alterations affecting the *PTEN* gene.

Paired normal and tumour genomic DNAs were purified from 41 colorectal, 29 gastric, 9 pancreatic and 38 endometrial carcinomas. We performed a pilot study of 10q-LOH, employing a PCR-based approach. LOH was identified in 6 (17%) of 35 informative colorectal cancers and 3 (15%) of 20 informative gastric tumours with three microsatellite markers surrounding *PTEN* (*D10S579, D10S215* and *D10S541*). Thirty-eight endometrial-cancer DNAs were assessed for LOH at three or more of the following seven loci: *D2S123, D9S162, D9S165, D10S215, D10S197, D10S541* and *D10S579*. Of 23 total informative endometrial-cancer samples, LOH was found in eleven cases (48%).

To determine whether intragenic *PTEN* DNA sequence alterations occurred in endometrial, colorectal, gastric and pancreatic cancers, PCR-SSCP analysis or direct DNA sequencing was performed for all exons and intron–exon boundaries. SSCP was performed as an initial screen on eighteen colorectal, eleven gastric and nine pancreatic cancers. Direct sequencing was performed on DNA samples from all thirty-eight endometrial cancers, an additional six 10q-LOH+ colorectal cancers and

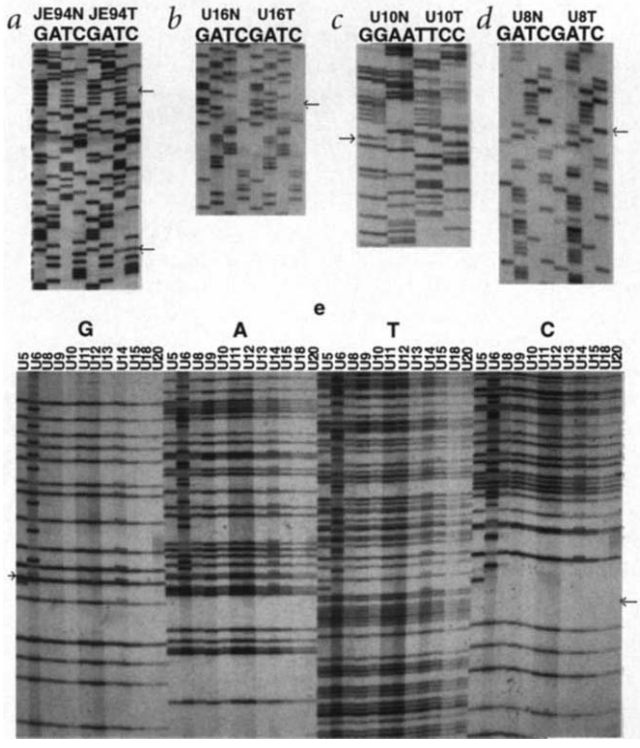

Fig. 1 *PTEN* mutations in endometrial cancers. ***a,b,d***, Nucleotide substitutions: somatic DNA sequence alterations are shown on the coding DNA strand in cases JE94N/JE94T, U16N/U16T and U8N/U8T. One C-to-T mutation at codon 233 and a T-to-A mutation at codon 250 are indicated in sample JE94T, one G-to-A substitution at the first base of intron 4 is shown in sample U16T and a C-to-A substitution is shown at codon 336 in sample U8T. Mutant bases are indicated by arrows. ***c,e***, Nucleotide deletions or insertions: a 4-bp deletion at codons 317–320 and a one-A insertion at codons 321–323 are indicated in samples U6T and U14T, respectively. A one-G deletion at codon 246 is shown in sample U10T. The arrow in *c* denotes the position of a one-G deletion in sample U10T (sense strand). The antisense strand sequence is shown in *e*. The arrow on the left in *e* indicates the position of a 4-bp deletion in sample U6T, while the arrow on the right indicates the position of a one-A insertion in sample U14T. Starting at each of these arrows, an upward displacement of the DNA sequence is visible in lanes G, A, T and C for tumours U6 and U14. As a first screening step, PCR-SSCP was performed according to methods described previously[9], with some modifications[10]. Primer sequences were modified from Steck *et al.*[6] and are available on request; PCR annealing temperature was 54 °C. For confirmation of insertion and deletion mutations, a simple PCR-based assay was also performed[11].