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DNA and Gene Amplification

Double minutes (DMs) and homogeneously stained regions (HSRs) provide good examples of phenomena that were originally regarded as cytological oddities but have turned out to be expressions of a fundamental process, called DNA or gene *amplification*. They arise almost exclusively in transformed and malignant cells. DMs were first described in the 1960s. They occur in most types of solid tumors and many leukemias (Chapter 27). Biedler and Spengler (1976) first described HSRs in human neuroblastoma cell lines and methotrexate-resistant hamster cell lines. HSRs are rare in human tumors but more common in cultured tumor cell lines (Benner et al., 1991).

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Double Minutes and Homogeneously Stained Regions

Double minutes are small paired chromatin structures that vary markedly in number and also in size, from very small double dots (Fig. 25.1a) to larger spherical structures and rings (Fig. 25.1b,c). They resemble tiny acentric chromosomes even by electron microscopy (Fig. 25.2; Jack et al., 1987). The lack of telomeric sequences and further molecular findings indicate that DMs contain circular DNA molecules (Lin et al., 1990; Von Hoff et al., 1990). DMs appear to replicate once in every mitotic cycle, during early S phase. Why, then, is their number so highly variable? The reason is that DMs lack centromeres, as shown, for example, by their lack of binding of antikinetochore antibodies (Haaf and Schmid, 1988). Consequently, their chromatids do not separate at anaphase. DMs tend to associate with centric chromosomes and travel at random towards either pole of the anaphase spindle, so one daughter cell may gain DMs and the other lose them. Other DMs lag behind, fail to be included in either daughter nucleus, and form micronuclei.

HSRs are interstitial or terminal additions or expansions of a chromosome arm that usually stain uniformly and rather lightly by any of the chromosome banding techniques (Fig. 25.1d). However, in rare cases an HSR has interspersed G- or C-bands, reflecting its heterogeneous origin (Cowell, 1982; Holden et al., 1987). HSRs, like DMs, generally replicate within a short period during early S phase. In most cases, they are located on the same chromosome arm as the native locus of the amplified DNA or gene, but sometimes they are present on a different chromosome or on multiple chromosomes. Since HSRs segregate normally in mitosis, they are more stable than DMs. However, they may increase or decrease in length and sometimes undergo other structural changes.

DMs and HSRs Are Expressions of Gene Amplification

Methotrexate (MTX) binds to and inactivates the enzyme dihydrofolate reductase (DHFR) and leads to cell death, especially of cancer cells. The development of resistance to MTX is accompanied by increased expression of DHFR and the appearance of HSRs. Observing this, Biedler and Spengler (1976) proposed that HSRs are expressions of gene amplification. Resistance to MTX or to any of

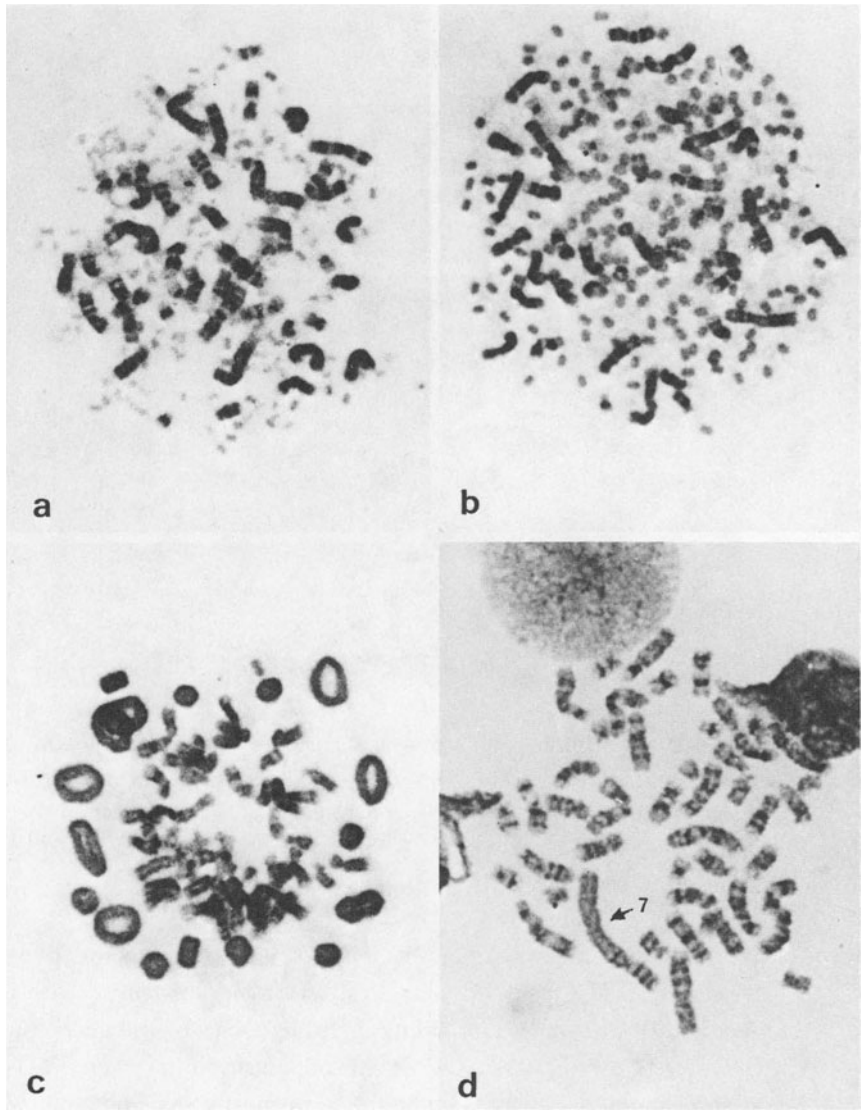

Figure 25.1. G-banded metaphase plates from a neuroblastoma cell line. (a) Small double minutes (DMs). (b) Medium-sized DMs. (c) Large rings. (d) Long homogeneously stained region on a chromosome 7 (Biedler JL, Ross RA, Shanske S, et al. 1980. Human neuroblastoma cytogenetics: search for significance of homogeneously stained regions and double minute chromosomes. In: Evans AE [ed] Advances in Neuroblastoma Research. Raven, New York, pp 81–96).

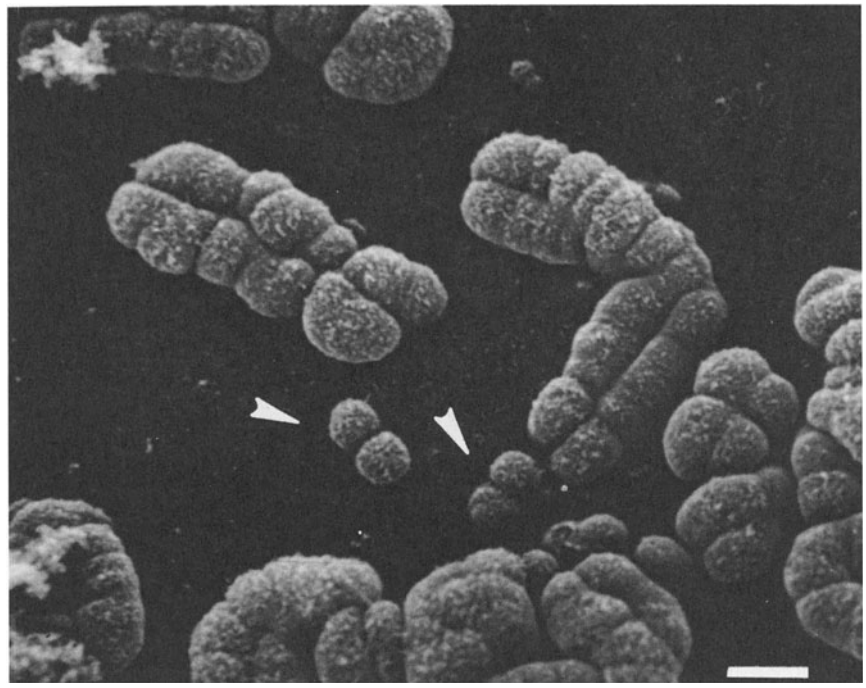

Figure 25.2. Scanning electron microscopy of normal and double minute (DM) chromosomes (arrows). The DMs vary in size, but their structure corresponds to that of normal chromosomes (reproduced from Jack et al., 1987; with permission of S Karger AG, Basel).

several other anticancer drugs, such as colchicine, vincristine, phosphonacetyl-L-aspartate (PALA), or actinomycin D, is usually associated with the development of DMs or HSRs containing multiple copies of the gene conferring drug resistance (Cowell, 1982). Oncogene amplification has been observed in many different kinds of cancer (Chapter 27). In most cases, the amplified genes are carried on extrachromosomal DMs or smaller submicroscopic circular extrachromosomal DNA molecules, called *episomes* or *amplisomes* (Pauletti et al., 1990). Amplified *MYC* oncogenes carried by episomes only a few hundred kb in size have been demonstrated by a variant of pulsed-field gel electrophoresis (PFGE) called field inversion gel electrophoresis (FIGE) in colon cancer and leukemia cell lines (Von Hoff et al., 1988). Standard gel electrophoresis separates, by size, fragments of DNA less than about 23 kb in size, but PFGE or FIGE will resolve fragments up to 1500 kb or larger (Cantor et al., 1988).

When cells containing DMs are treated with hydroxyurea, budding of the nuclear membrane and micronucleus formation occur. Acentric episomes and DMs are preferentially included in the micronuclei, leaving the cells with fewer DMs, fewer copies of the amplified oncogene, and reduced tumorigenicity. This also provides a mechanism for easy isolation and identification of an amplified gene (Shimizu et al., 1998). This can also be done by comparative genomic hybridization (Chapter 8), which has been used to detect and map amplified DNA sequences in breast cancer (Kallioniemi et al., 1994). An alternative approach is microdissection of the DMs and PCR amplification of the microdissected DM DNA to yield a probe for FISH or other molecular analysis. Findings with this approach have confirmed the origin of DMs in the HL-60 leukemia line from a single chromosomal site, 8q24.1–q24.2, where the *MYC* gene resides (Sen et al., 1994).

In some cases, DMs and HSRs are interconvertible. In the earliest passages of HL-60 leukemia cells, amplified MYC oncogenes are present on submicroscopic amplisomes. At later passages, these have been replaced by DMs, and at still later passages the amplified *MYC* genes have shifted to a chromosomal site (Von Hoff et al., 1990). Integration of DMs into random chromosomal sites to yield an HSR appears to be a common event. HSRs can sometimes give rise to DMs, and one mechanism for this has been identified, as described below (Toledo et al., 1992).

Normal cells rarely develop drug resistance by gene amplification (Livingstone et al., 1992). A 100-fold amplification of the CHE (cholinesterase) gene was observed in a farmer whose parents were exposed over many years to an organophosphate. A son and a grandchild inherited the CHE amplicon (Prody et al., 1989). A father and two of his sons, all rapid metabolizers of the antihypertensive drug debrisoquine had a 12-fold amplification of the cytochrome P450 *CYP2D* gene, whose product metabolizes the drug (Johansson et al., 1993). HSRs containing amplified rRNA genes (Miller et al., 1978; Cowell, 1982) or GACA repeats (Schmid et al., 1994) have been observed in a number of individuals or families. Some fragile sites may arise by amplification of minisatellites. For example, the *FRA3B* fragile site is an amplified AT-rich minisatellite (Chapter 20).

Why should DNA amplification be so rare in normal cells? One explanation is that there are genes that suppress amplification. Cancer cells support a high level of amplification, but when they are fused with normal cells, the hybrids are unable to amplify, suggesting that the cancer cells lack the product of a gene that suppresses amplification (Tlsty et al., 1992). The *TP53* protooncogene is a

likely candidate, because cancer cells often have nonfunctional p53 protein due to *TP53* mutations (Chapter 27). Cells homozygous for a nonfunctional *TP53* allele are capable of amplifying a gene, whereas cells heterozygous for the mutant allele are, like normal cells, incapable of gene amplification (Livingstone et al., 1992). The introduction of wild-type p53 into cells with nonfunctional p53 alleles inhibits gene amplification (Yin et al., 1992). Thus, the normal *TP53* gene product suppresses amplification. To understand how p53 prevents amplification, one must first learn how amplification is brought about.

Mechanisms of Gene Amplification

Gene amplification arises by a variety of mechanisms, most of them poorly understood. Extrachromosomal copies of a gene may arise either by double-strand breakage in a stalled replication bubble, followed by recombination to form a circular molecule (Windle and Wahl, 1992), or by looping out and excision from one homologue of a DNA segment that includes the gene (Coquelle et al., 1997). The breakpoints and amplisome size depend upon the local chromatin environment of the gene. The breakpoints that lead to *DHFR*-containing DMs tend to occur 500 kb upstream and 200 kb downstream of the *DHFR* gene, very close to unmethylated CpG clusters, or *islands* (Foureman et al., 1998). CpG islands are usually found at the promoter region of housekeeping genes and are in an open chromatin conformation that is readily accessible both to transcription factors and to nucleases. Nuclease-sensitive sites are implicated in *MYC* gene amplification (Razin et al., 1995). The p53 protein is essential for DNA damage checkpoint function (Chapter 26). Therefore, *TP53* mutations would greatly increase the probability that the breaks would not be repaired and an extrachromosomal amplisome could arise. Initially, a single copy of the amplified gene is carried by each amplisome, but DMs tend to have dimers or larger numbers of the gene, arranged as either head-to-tail direct repeats or head-to-head inverted repeats (Fakharzadeh et al., 1993). Further increases in copy number are brought about by the unequal random segregation of DMs at anaphase.

Intrachromosomal amplification may arise at a single step by multiple cycles of unscheduled DNA replication at a single locus, followed by multiple recombination events that link individual units together and to the chromosome (Roberts et al., 1983). Windle and Wahl (1992) favor a modified version of this onionskin model in which the initiating event is the occurrence of double-strand

breaks (DSBs) in stalled replication bubbles. This is followed by amplification and recombination, leading to extrachromosomal DMs or intrachromosomal HSRs. The DMs appear to be able to integrate almost at random into any chromosome. This mechanism may account for the presence of the tandemly repeated copies of the *N-MYC* oncogene observed in HSRs in neuroblastomas, in which the HSRs are on different chromosomes from the one with the native *N-MYC* locus (Amler et al., 1992). However, it is unlikely that HSRs generally arise by integration of DMs or smaller episomal amplicons. In most cases HSRs are found on the same chromosome arm as the native site of the unamplified gene, and the unit of intrachromosomal amplification is usually much larger than the size of DMs, especially early in the process.

A second mechanism for intrachromosomal amplification is unequal homologous exchange between sister chromatids, which is also responsible for the well-known variations in the number of tandemly repetitive rRNA genes and satellite DNAs. It probably accounts for the occasional amplification of rRNA genes (Miller et al., 1978). In fact, unequal SCEs have been observed in a melanoma cell line in which the HSRs contained both amplified ribosomal RNA genes and another tandemly repetitive element (Holden et al., 1987). While unequal homologous exchanges increase or decrease the copy number of tandem repeats, they do not account for DMs or for the initial amplification event needed to produce the tandem repeats of a single-copy gene necessary for unequal exchange to operate.

Cowell and Miller (1983) proposed a novel explanation for intrachromosomal amplification, suggested by their study of a rapidly evolving HSR. Their hypothesis was that the initial event leading to DNA amplification is telomere loss, leading to the formation of a dicentric chromosome, which undergoes anaphase bridge-breakage-fusion-bridge cycles, leading to amplification. This mechanism gained support from the finding that the generation of a large inverted duplication is an early event in the amplification process (Ford and Fried, 1986). Furthermore, it provides an explanation for the later observation that the unit of amplification is initially quite large but decreases with time (see, for example, Toledo et al., 1992).

The telomere loss/bridge-breakage-fusion-bridge cycle hypothesis has gained further support from observations on telomere length and chromosome instability. In sperm, the TTAGGG repeats at the telomeres of the various chromosomes are at their maximum length, 10–14 kb. In somatic cells, which lack telomerase, the telomeres are several kb shorter and quite heterogeneous in length. In primary tumors and some tumor cell lines, telomeres are even shorter,

down to as little as 5 kb or so (de Lange et al., 1990). When cultured diploid cells are infected with a transforming DNA virus, such as SV40, the telomeres get even shorter, down to an average of 1.5 kb, and dicentric chromosomes become abundant, setting the stage for the bridge-breakage-fusion-bridge cycles that can lead to amplification. If the cells turn on their telomerase gene, telomere length stabilizes and the cells are immortalized (Counter et al., 1992). Ironically, amplification is one mechanism by which this is achieved. Thus, amplification may play a general and very important role in carcinogenesis, since most cancers show amplification of the gene for the telomerase RNA gene (Soder et al., 1997).

Telomere loss can also occur as a result of chromosome breakage, and that is probably why fragile sites play such an important role in intrachromosomal amplification: They are hotspots for the chromosome breakage that is involved in generating amplifiable structures (Coquelle et al., 1997). Some environmental agents may be carcinogenic because they induce breakage at fragile sites. Cigarette smokers tend to show an increase in breaks at several fragile sites and also at the sites of several oncogenes, such as *BCL1* (Kao-Shan et al., 1987). The various drugs that induce drug resistance through intrachromosomal amplification also break chromosomes and, through the formation of dicentric chromosomes, trigger bridge-breakage-fusion-bridge cycles that lead to amplification. Intrachromosomal amplification of *DHFR* and *AMPD2* (adenylate [adenosine mono phosphate] deaminase 2) genes is associated with the presence of breaks at nearby fragile sites telomeric to the locus of each gene. In some cases, the amplified region is flanked by two fragile sites. The telomeric one is important in generating a dicentric chromosome, while the one on the centromeric side, which arises in a subsequent bridge-breakage-fusion-bridge cycle, defines the size of the unit of amplification; this may include several genes from a region. For example, fragile sites *FRA11F* at 11q14.2 and *FRA11A* at 11q13.3 flank the *BCL1* (B cell CLL/lymphoma), *cyclin D1*, and fibroblast growth factor genes *FGF3* and *FGF4* in 11q13, which are coamplified in breast, head and neck, and other carcinomas (Coquelle et al., 1997). As the names imply, several of the coamplified genes are oncogenic, and their joint amplification may add to the malignancy of the cells.

The most exciting new insight into gene amplification has come from a two-color FISH study. Toledo et al. (1992) coamplified the *AMPD2* gene and the *P3C4* marker, 5 Mb away. Analysis of metaphase chromosomes showed an HSR containing long arrays of inverted repeats, with copies of the two genes interspersed throughout. This is best explained by bridge-breakage-fusion-bridge

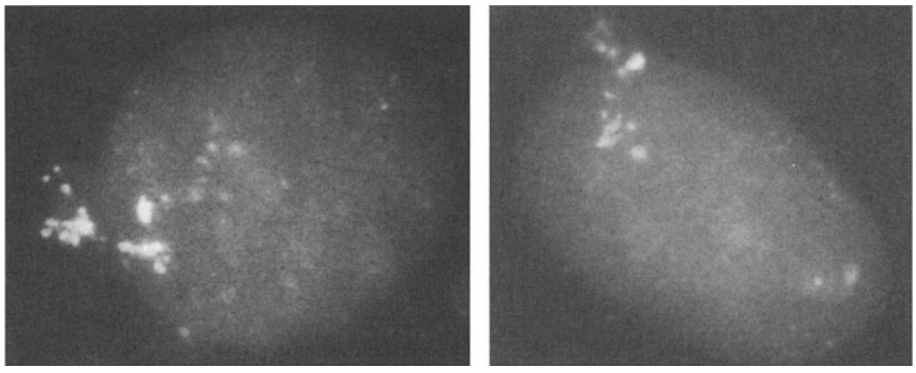

Figure 25.3. Preferential segegation of one amplified marker into nuclear blebs containing *P3C4* only (left) or *AMPD3* only (right) (reproduced from Toledo et al., EMBO J 11:2665–2673, 1992, with permission of Oxford University Press) (See color insert).

cycles. The really novel findings came from an examination of interphase cells. In these, the amplified *AMPD2* genes were clustered in one nuclear domain and the *P3C4* genes in a different domain, at a different point at the nuclear envelope. Sometimes one of the clusters was extruded from the nucleus, forming a micronucleus. Surprisingly, each micronucleus usually contained only one of the two types of genes (Fig. 25.3; see color insert). This clearly indicates that multiple chromosome breaks have taken place (presumably in interphase), with selective loss of one type of gene from the amplified region and retention of the other (Fig. 25.4). Micronucleus formation of this type has been seen in at least one other amplifying line (Smeets et al., 1994), so this may be a general mechanism for shortening the unit of amplification while retaining a selectable drug-resistance or oncogenic marker. As a corollary, one might predict that genes that continue to be coamplified must confer a selective growth advantage over those containing a single amplified gene (Coquelle et al., 1997).

Nuclear protrusions, or blebs, have long been observed in cancer cells and were thought to depend upon the presence of a very long chromosome arm that simply trailed behind at anaphase and protruded at interphase. However, their formation most often reflects the presence of an amplified region that is undergoing reorganization. The amplified sequences enclosed by such projections are frequently detected in the micronuclei that arise from them (Pedeutour et al., 1994). The findings of Toledo et al. (1992) show how complex the evolution of HSTs and DMs can be. The massive breakage associated with the selective

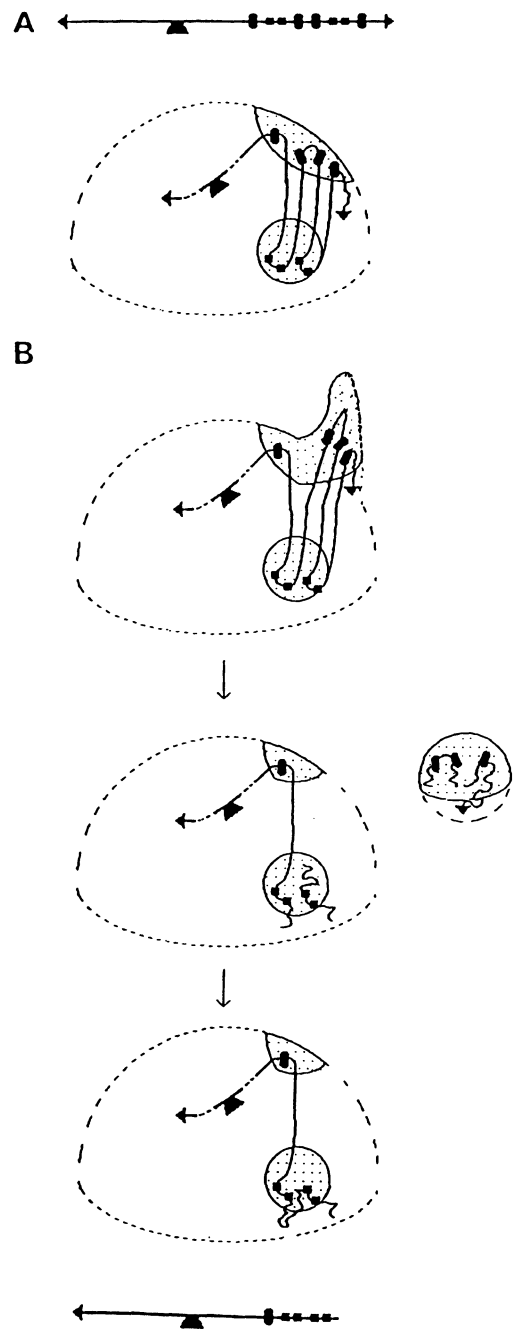

Figure 25.4. (A) Metaphase diagram showing two amplified interspersed markers, *P3C4* and *AMPD3*. (B) Diagrams showing preferential clustering of these markers into separate nuclear areas, followed by formation of a nuclear bleb containing the extra copies of one marker and their sequestration into a micronucleus. After repair of the multiple breaks, the remaining nuclear amplified unit is shorter and enriched for the other marker (reproduced from Toledo et al., EMBO J 11:2665–2673, 1992, with permission of Oxford University Press).

extrusion of amplified copies of an interspersed marker leaves the amplified gene copies on short DNA fragments. This could lead to the formation of either an HSR in which the copies of the gene are now considerably closer to each other than they were before, or DMs. This may be the mechanism by which an HSR is replaced by DMs.

How Does Gene Amplification Lead to Cancer?

In many cancer cells, one or more cellular oncogenes are amplified and the gene product is overexpressed. This leads to unregulated cell proliferation and plays an important role in carcinogenesis, as described in Chapter 27. A second mechanism is blocking of programmed cell death (apoptosis). Many cancer cells produce antigens that are recognized by the body's immune system. This leads to cell killing by activated T lymphocytes or natural killer lymphocytes, which produce a protein called Fas ligand (FasL). FasL induces apoptosis in the target cells by binding to the cell death receptor Fas. Nearly half of 35 lung and colon cancers were found to have 2- to 16-fold amplification of the gene for a decoy receptor, called DcR3, that binds FasL and prevents it from binding to Fas. This blocks cancer cell killing by the body's immune surveillance mechanism (Pitti et al., 1998).

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