

Transcriptional regulation of the *H-ras1* proto-oncogene by DNA binding proteins: mechanisms and implications in human tumorigenesis

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Summary

Altered expression of *ras* genes is a common event in human tumors. Transcriptional regulation of the *H-ras1* proto-oncogene occurs through nuclear factors that recognize elements in the promoter region of the gene, in the first and fourth intron and in the VTR unit and involves alternative splicing and specific methylation patterns, as well. Aberrant levels of the Ras p21 protein are detected in a variety of human tumors and are often correlated with clinical and prognostic parameters. Thus, understanding the regulation of the expression of *ras* genes provides a useful target for gene therapy treatments.

I. Introduction

ras genes are a ubiquitous eukaryotic gene family. They have been identified in mammals, birds, insects, mollusks, plants, fungi and yeasts. Their sequence is highly conserved, thus revealing the fundamental role they play in cellular proliferation (Spandidos, 1991).

A. The structure of *ras* genes

Three functional *ras* genes have been identified and characterized in the mammalian genome, *H-ras1*, *K-ras2* and *N-ras*, as well as two pseudogenes, *H-ras2* and *K-ras1* (Barbacid, 1987). All three *ras* genes have a common structure with a 5' non-coding exon (exon -I) and four coding exons (exons I-IV). The introns of the genes differ widely in size and sequence, with the coding sequences of human *K-ras* spanning more than 35 kb, while those of *N-ras* and *H-ras* span approximately 7 and 3 kb, respectively. The *K-ras* gene has two alternative IV coding exons, thus encoding two proteins, K-RasA and K-RasB (McGrath et al, 1983), with the K-RasB form being more abundant. The *H-ras* gene also has an alternative exon in the fourth intron (Cohen et al, 1989). In addition, *H-ras* has a variable tandem repeat sequence (VTR), located downstream of the polyadenylation signal, which exhibits enhancer activity (Spandidos and Holmes, 1987, Cohen et al, 1987).

B. Ras proteins: structural characteristics and function

The H-Ras, N-Ras and K-RasA proteins are 189 amino acids long, whereas K-RasB is shorter by one amino acid. They all have a molecular weight of 21kDa and are termed p21 proteins. The p21 proteins are identical at the 86 N-terminal amino acid residues, they possess an 85% homology in the next 80 amino acid residues and diverge highly at the rest of the protein molecule, with the exception of the four C-terminal amino acids which share the common motif CAAX-COOH (C, Cysteine 186; A, Aliphatic amino acid-Leucine, Isoleucine or Valine; X, Methionine or Serine) (Lowy and Willumsen, 1991). The Ras protein is synthesized as pro-p21, undergoes a series of post-translational modifications at the C-terminus increasing the hydrophobicity of the protein and associates with the inner face of the plasma membrane. Sequences at the C-terminus are essential for membrane association and the conserved Cys 186 is required to initiate the post-translational modifications of pro-p21 (Willumsen and Christensen, 1984).

The superfamily of Ras proteins comprises a group of small GTPases, regulating an astonishing diversity of cellular functions (Makara et al, 1996). They are located at the heart of a signal transduction pathway that links cell-surface receptors through a protein kinase cascade to

changes in gene expression and cell morphology and to cell division mechanisms. The Ras p21 protein interacts directly with the Raf oncoprotein to recruit the MAP kinases and their subordinates, thus converting a mitogenic signal initiated by membrane receptors with tyrosine kinase activity to a cascade of Serine/ Threonine kinases with multiple targets, including cytoskeleton, transcription factors, inflammatory mediators and other kinases (Avruch et al, 1994, Marshall, 1995).

C. *ras* oncogenes: mechanisms of activation

The *ras* family of proto-oncogenes, is a frequently detected family of transformation-inducing genes in human tumors. Implication of *ras* genes in human tumorigenesis occurs by four different mechanisms: point mutations (Kiaris and Spandidos, 1995), gene amplification (Pulciani et al, 1985), insertion of retroviral sequences (Westaway et al, 1986) and alterations in regulation of transcription (Zachos and Spandidos, 1997). With the exception of mutations, all other mechanisms result in activation of the transforming properties of *ras* genes by quantitative mechanisms.

The c-*H-ras1* gene is the best studied member of the family and provides a good example for understanding the mechanisms of gene regulation. The *H-ras* proto-oncogene expression is regulated by elements located in the promoter region, in intronic sequences and in the 3' end of the gene. In addition, *H-ras* gene expression is regulated by alternative mechanisms such as DNA methylation and alternative splicing (reviewed by Zachos and Spandidos, 1997). Alterations in the *H-ras* expression levels are a common mechanism of human tumorigenesis.

II. Transcriptional regulation of the *H-ras* gene from promoter-like sequences

The *H-ras* gene promoter contains multiple RNA start sites, multiple GC boxes and has no characteristic TATA box (Ishii et al, 1985). These features are characteristic of housekeeping genes. Most promoter region studies have focused on the region upstream of the 5' splice site of the first intron of the gene (nucleotides 1-577), although others consider the SstI fragment (nucleotides 1-1054) that encompasses a part of the first intron as well, to be the gene promoter (Spandidos et al, 1988).

Regulation of gene expression depends on a variety of nuclear factors (Boulikas, 1994). A great number of regulatory elements in the *H-ras* promoter has been reported, but the results were often controversial, depending on the followed experimental procedure. Transcription factors that interplay on the regulatory regions of the *H-ras* gene promoter include Sp-1, NF-1, AP-1 and some unknown factors as well.

The Sp-1 is a mammalian DNA binding protein activating transcription by interacting through zinc finger domains with guanine-rich DNA sequences called GC

boxes (Berg, 1992). The transcription factor AP-1 is the nuclear factor required to mediate transcription induced by phorbol ester tumor promoters and recognizes a short TGACTCA sequence (Lewin, 1991). Both c-Jun, encoded by members from the *jun* family (*jun*, *junB*, *junD*), and c-Fos proteins are active components and contribute to the activity of AP-1 by forming c-Jun homodimers as well as c-Jun-c-Fos heterodimers. In addition, there appears to be a mutual antagonism between activation by AP-1 and glucocorticoid receptors at target genes that contain recognition sites for both factors, via protein-protein interactions (Yang-Yen et al, 1990). Finally, the NF-1 (CTF) nuclear factor binds the CCAAT element (CAAT box) and is involved in both gene transcription and DNA replication. The NF-1 C-terminal region is proline rich and activates transcription through interference with the transcription machinery (Mermod et al, 1989).

Ishii et al (1986), identified six GC boxes that bind the Sp-1 transcription factor as the essential regulatory elements within the *H-ras* promoter. Using deletion analysis of the *H-ras* promoter region by focus formation assay in NIH 3T3 cells, Honkawa et al (1987) reported a minimum promoter region of 51 bp length, which was GC rich (78%) and contained a GC box. Lowndes et al (1989) located a 47 bp element, distinct of the one reported by Honkawa et al, that upregulated the transcriptional activity of the promoter region by 20- to 40-fold and contained a GC box and a CCAAT box, binding the NF-1 (CTF) factor. Transient expression assays in which a series of mutants spanning the promoter region of *H-ras* were ligated to a promoterless chloramphenicol acetyl transferase (CAT) vector, were used in this analysis. Jones et al (1987), also identified two NF-1 binding sites, one strong, also noted by Honkawa et al, and one weak. Trimble and Hozumi (1987), using CAT transfection experiments in CV-1 cells, identified a 100 nucleotide region, encompassing the consensus CCAAT box and two Sp-1 sites. However, Nagase et al (1990), using deletion mutants in CAT assays in CV-1 and A-431 cells, suggested that the presence of Sp-1 binding sites at specific positions may not be essential for promoter activity, but a number of Sp-1 binding sites in the region could be required. Lee and Keller (1991), transfected recombinant plasmids encompassing internal deletions and point mutations of the promoter region in HeLa cells and performed CAT assays. They reported a GC box, an unidentified element and a new element CCGGAA directly upstream the GC box, as the most important regulatory elements. Spandidos et al (1988), using recombinant plasmids in CAT activity experiments showed that AP-1-like proteins participate in control of *H-ras* transcription and identified four TPA responsive-AP-1 binding elements in the *H-ras* promoter.

A great variety of the transcription initiation sites was also identified (Lowndes et al, 1989, Nagase et al, 1990,

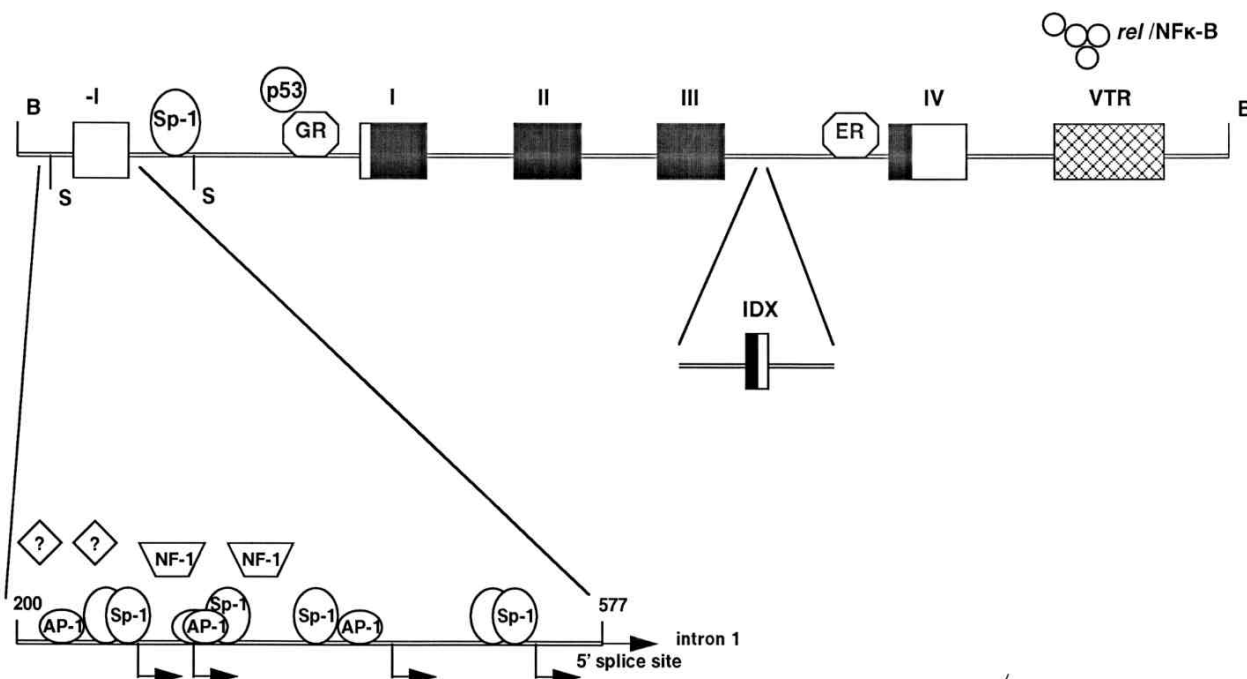


Figure 1. A synopsis of the reported nuclear factors participating in *c-H-ras1* transcriptional regulation. Factors recognize sequences in the *H-ras* promoter, in intronic sequences and in the VTR region. Exons, rectangles; coding sequences, filled rectangles; VTR, cross-hatched box; B, BamHI cleavage site; S, SstI cleavage site; arrows, major transcriptional initiation sites; ?, unknown regulatory factors; IDX, intron D exon.

Lee et al, 1991) using S1 nuclease analysis. A synopsis of the reported nuclear factors that participate on the promoter activity of *H-ras*, and of the major RNA start sites, are shown in **Fig. 1**.

III. Regulation of the *H-ras* gene expression from intronic sequences

Intronic sequences play an important role in *H-ras* regulation. The nuclear factor Sp-1, steroid hormone receptors and the P53 onco-suppressor protein recognize sequences in the first and fourth introns of the *H-ras* gene.

A. The Sp-1 box

There is evidence that the mutant T24 *ras* 0.8 kb SstI DNA fragment is a more potent activator of gene expression, compared to the corresponding normal *H-ras* fragment (Spandidos and Pintzas, 1988). A structural basis for this difference was shown to be a 6 bp element in the mutant *H-ras* fragment, that was absent in the normal *H-ras*, in the first intron of the gene. This element was proved to contain an Sp-1 binding site (Pintzas and Spandidos, 1991) (**Fig. 1**).

B. The Hormone response elements (HREs)

Steroid hormone receptors produce an enormous number of biological effects in different tissues as hormone activated transcriptional regulators (Beato, 1989, Beato et al, 1995). Such a process requires the coordinate expression of multiple genes and likely candidates are signal transducers, capable of secondarily controlling the transcription of sets of genes that lack steroid hormone response elements. Proto-oncogenes are theoretically well suited for this role, as they exhibit precise temporal patterns of expression during proliferation and differentiation, they participate in signal transduction and regulate the expression of multiple genes in a cascade fashion (Bishop, 1991). Thus, they may integrate signals from steroids and other regulatory factors and amplify the cellular response to the hormone. Evidence for steroid hormone regulation of proto-oncogenes encoding for nuclear transcription factors has already been provided for *c-fos*, *c-jun* and *c-myc* (Hyder et al, 1994).

1. Regulation of *c-H-ras* by steroid hormone receptors

Zachos et al (1995), identified sequences in the 3' end of the first intron and in the fourth intron of the *H-ras*

gene, with high similarity with the glucocorticoid response element (GRE) and estrogen response element (ERE) consensus oligonucleotides, respectively (**Fig. 1**). Using nuclear extracts from human and murine cell lines in gel retardation assays, it was shown that both glucocorticoid receptor (GR) and estrogen receptor (ER) specifically recognize the corresponding *H-ras* elements (Zachos et al, 1995).

H-ras p21 protein, is a G-protein involved in signal transduction beginning from transmembrane growth factor and peptide receptors with tyrosine kinase activity (Avruch et al, 1994, Marshall et al, 1995). Steroid receptors, participate in distinct signalling mechanisms, involving transcriptional regulation of genes by ligand activated receptors (Beato, 1989, Beato et al, 1995). Thus, hormone regulation of *H-ras* provides evidence for a direct interaction of these two pathways, allowing the cell to have an additional regulatory "switch" by hormonally modulating the levels of G-proteins at the transcriptional level (Zachos et al, 1996a).

Moreover, the first intron of the gene contains well conserved regions between human and rodents (Hashimoto-Gotoh et al, 1988) and encompasses positive and negative elements influencing *H-ras* expression, possibly at post-transcriptional level (Hashimoto-Gotoh et al, 1992). It is noteworthy that the GRE, as well as the p53 element that will be discussed later, are located in the conserved region of the intron, thus providing evidence for an essential role in regulating *H-ras* expression. Moreover, the *H-ras* GRE is located within the first positive element.

2. Interaction of the H-ras and steroid hormone receptors in gynecological cancer

The human endometrium and ovary are major targets for action of glucocorticoids. Sex hormones and steroids act as tumor promoters. The level of receptor binding in *H-ras* hormone response elements was examined in gel retardation assays, using nuclear extracts from human endometrial and ovarian lesions and from adjacent normal tissue (Zachos et al, 1996b). Increased binding of the glucocorticoid receptor in *H-ras* GRE was observed in more than 90% of endometrial and in all ovarian tumors tested, compared to the adjacent normal tissue. Moreover, elevated binding of the estrogen receptor in *H-ras* ERE was found in all pairs of ovarian tumor/ normal tissue examined (Zachos et al, 1996b). Thus, it was proposed that *H-ras* is implicated in human gynecological lesions through elevated steroid receptor binding.

In addition, previous data showed cooperation of the *H-ras* with steroids in cell transformation (Kumar et al, 1990) and overexpression of the Ras p21 in ovarian tumors, compared to normal or benign tumor tissue (Katsaros et al, 1995). By combining these data, it was proposed that the high levels of steroid and sex hormones in human genital tract may result in increased amounts of ligand activated steroid receptors. Furthermore, receptors bind to the *H-ras* DNA and induce elevated transcription of the *H-ras* oncogene, resulting in an increased oncogenic potential.

Thus, endometrial and ovarian epithelial cells may have a predisposition to develop neoplastic abnormalities in addition to a second tumorigenic event, e.g. viral infection, loss of an onco-suppressor gene, or mutational activation of a proto-oncogene (Zachos et al, 1996a).

C. The p53 element

One of the major roles of wild-type P53 onco-suppressor protein is to trigger cell cycle arrest or apoptosis in response to DNA damage by acting as a sequence specific transcription factor that binds to DNA and activates genes involved in the control of the cell cycle, including p21, *gadd45*, *bax*, *mdm2* and PCNA (Zambetti et al, 1993, Hainaut, 1995). The mutant forms of P53 promote tumorigenesis by dominant-negative inhibition of wild-type P53 through cross-oligomerization. Moreover, P53 mutants were proved to exert oncogenic functions of their own (Dittmer et al, 1993).

1. Regulation of the c-H-ras by the P53 tumor-suppressor protein

H-ras contains within its first intron sequences that partially match the p53 consensus binding site (**Fig. 1**). Using gel retardation assays it was shown that wild-type P53, as well as the "hot spot" mutant His 273 recognize the *H-ras* element with high affinity (Spandidos et al, 1995, Zoumpourlis et al, 1995). Furthermore, the *H-ras* element functioned as a P53-dependent transcriptional enhancer in the context of a reporter plasmid, thus suggesting that P53 is a physiological regulator of *H-ras* expression (Spandidos et al, 1995).

Activation of *H-ras* expression by P53 may seem a paradox, since p53 is a tumor suppressor and *H-ras* a proto-oncogene. However, precedence has already been established for activation of proto-oncogenes by P53. Wild-type P53 induces expression of *mdm-2*, whose protein product inhibits the tumor suppressor activities of P53 and Rb (Xiao et al, 1995). Interestingly, there are certain similarities in the organization of the p53 elements of the *H-ras* and *mdm-2* genes, which are not shared with the p53 elements of other genes targeted by P53. In *H-ras* there are three half sites: two of them are contiguous, while the third is 8 nucleotides upstream. In *mdm-2* there are again three half-sites: two are contiguous, while the third one is located 28 nucleotides upstream (Wu et al, 1993). In contrast to *H-ras* and *mdm-2*, the elements of the other genes regulated by P53 are contiguous. The organization of the half-sites affects the ability of the P53 protein to recognize these elements. Wild-type P53 reversibly switches between two conformations: the "inactive" T state, with dihedral symmetry, which can recognize only non-contiguous half-sites and the "active" R state, which can recognize even contiguous half-sites (Waterman et al, 1995). Thus, it is suggested that *H-ras* and *mdm-2* genes allow regulation by even the "inactive" T state of P53 protein.

The H-*ras* p53 element is located within the first intron. Interestingly, the p53 element of the *mdm-2* is also located in the first intron of the gene (Wu et al, 1993). The significance of this is not understood at this time. The *mdm-2* has an internal promoter in the first intron. Transcripts initiating at both promoters contain the entire protein sequence, however they differ in the efficiency with which translation is initiated in codon 1. Thus, transcripts that include the first exon mostly express an N-terminally truncated Mdm-2 protein, whereas transcripts from the internal promoter express a full-length protein. P53 induces expression only from the internal promoter and only the full-length form can associate with P53, closing the autoregulatory feedback loop (Barak et al, 1994). It remains to be determined whether P53 induces expression of transcripts initiating at the first intron of H-*ras*, as well as the biological significance of such transcripts.

The p53 tumor suppressor may therefore exert its cellular effects by coordinate activation of genes that suppress and induce cell proliferation.

2. Altered binding of p53 protein to the H-*ras* element in human tumors

Mutation and overexpression of the p53 tumor suppressor is a common event in human endometrial and ovarian cancer (Berchuck et al, 1994) and is associated with poor prognosis (Levesque et al, 1995, Kihana et al, 1995). Moreover, aberrant regulation of the H-*ras* gene expression also participates in the development of human gynecological lesions (Zachos and Spandidos, 1997).

Using nuclear extracts from human endometrial and ovarian tumors and from the adjacent normal tissue in gel retardation assays, we examined the binding levels of the P53 protein to the H-*ras* element (our unpublished results). Elevated P53 binding in the tumor tissue was found in 5/11 (45%) of endometrial and in 2/5 (40%) of ovarian cases. Loss of P53 DNA binding activity was observed in 3/11 (27%) of endometrial and in 1/5 (20%) of ovarian tumors. In the remaining 3/11 (27%) of endometrial and in 2/5 (40%) of ovarian pairs tested, no alteration in the P53 binding levels was observed. In order to interpret the results, all pairs were subsequently tested for mutations in exons 4-9 of the p53 gene using PCR-SSCP analysis. No mutation was observed in any case showing elevated DNA binding activity, thus implying for overexpression of the wild-type p53 gene in these tumors. In addition, no p53 mutational alteration was observed in the cases showing similar DNA binding levels in tumor versus the adjacent normal tissue. However, a mutated allele was detected in all four endometrial and ovarian cases showing loss of P53 DNA binding activity. We therefore suggest that P53 could directly modulate the H-*ras* oncogenic potential in human endometrial and ovarian lesions, depending on the expressed levels of P53 and the status of the protein (wild-

type or mutated forms), thus providing additional evidence for the role of H-*ras* in human carcinogenesis.

IV. The role of the VTR

Variable tandem repeats (VTRs, minisatellites) are highly polymorphic structures characterized by the tandem repetition of short (up to 100 bp) sequence motifs. Several observations on tandemly-repetitive elements within viral genomes (Yates et al, 1984) have led to the speculation that some human minisatellites might serve as regulatory regions for cellular transcription or replication.

A. The H-*ras* minisatellite sequence as transcriptional enhancer

The human H-*ras* gene contains a VTR region located 1 kb upstream the polyadenylation signal (**Fig. 1**). It consists of 30 to 100 copies of a 28 bp consensus repeat. Four common alleles and more than 25 rare alleles have been described (Krontiris et al, 1993). It was shown that the H-*ras* VTR sequences possess endogenous enhancer activity, independently from orientation, however this activity is promoter specific (Spandidos and Holmes, 1987, Cohen et al, 1987). The 28 bp repeat unit of the minisatellite binds four proteins (p45, p50, p72 and p85) which are members of the *rel*/NF- κ B family of transcriptional regulatory factors (Trepicchio and Krontiris, 1992).

B. VTR rare alleles of the H-*ras* and ovarian cancer risk

Women who carry a mutation in the *BRCA1* gene have an 80% risk of breast cancer and a 40% risk of ovarian cancer by the age of 70 (Easton et al, 1995). The varying penetrance of *BRCA1* suggests a role for other genetic and epigenetic factors in tumorigenesis of these individuals. H-*ras* was the first example of a modifying gene on the penetrance of an inherited cancer syndrome. Rare alleles of the H-*ras* VTR locus duplicate the magnitude of ovarian cancer risk for *BRCA1* carriers, but not the risk for developing breast cancer (Phelan et al, 1996). It was suggested that H-*ras* VTR alleles show differences in modulating gene transcription, that H-*ras* VTR alleles are in linkage disequilibrium with other genes important in tumorigenesis, or that rare alleles provide a marker for genomic instability (Phelan et al, 1996).

V. The role of the DNA methylation status

DNA methylation is essential for embryonic development and alterations in the DNA methylation status are common in cancer cells. CpG sites in vertebrates are either clustered in 0.5-2 kb regions called

Table I. *ras* gene overexpression in human tumors and correlation with clinical parameters.

Tumor type	Frequency (%)	<i>ras</i> gene	Stage in tumorigenesis	Prognosis of the disease
Neuroblastoma	50-80	H-, <i>ras</i>	early	favourable
Head and neck	54	H-, K-	early	favourable
Esophagus	40	H-	unknown	unknown
Larynx	57-86	H-, K-, N-	unknown	unknown
Thyroid	85	<i>ras</i>	early	unknown
Lung	64-85	<i>ras</i>	late	poor
Liver	60	<i>ras</i>	unknown	unknown
Small intestine	70	<i>ras</i>	unknown	unknown
Stomach	35	K-, <i>ras</i>	late	poor
Pancreas	42	<i>ras</i>	unknown	unknown
Colon	31	H-, K-, <i>ras</i>	early	poor
Breast	65-70	<i>ras</i>	unknown	unknown
Bladder	39-58	H-, K-, N-	early	poor
Endometrium	18-95	<i>ras</i>	late	unknown
Ovary	45	<i>ras</i>	late	poor
Leukemias	39-67	H-, K-, N-	unknown	unknown

CpG islands, or are dispersed, in which case they are mostly methylated and constitute mutational hotspots (Jones, 1996). The CpG islands are associated with gene promoters (e.g. *H-ras*) or coding regions (e.g. *p16*) and are unmethylated in autosomal genes. 5' Methyl-cytosine can affect transcription by altering the DNA binding activities of transcription factors. This could be done either directly, for example binding of *trans*-acting proteins at AP-2 sites is inhibited (Comb and Goodman, 1990), or indirectly, by enhanced binding of methylated DNA binding protein (MDBP) which stereochemically inhibits DNA binding of transcription factors (Boyes and Bird, 1991).

The promoter region of the *H-ras* gene is hypomethylated in human tumors compared to the corresponding normal tissue (Feinberg and Vogelstein, 1983). Furthermore, methylation of *cis*-elements decreases *H-ras* promoter activity *in vitro* (Rachal et al, 1989) and inhibits the transforming activity of the oncogene (Borello et al, 1987). It is therefore suggested that epigenetic and reversible mechanisms, like DNA methylation, can regulate the expression of proto-oncogenes and silence genetically activated human oncogenes.

VI. Differential expression of the *H-ras* gene is controlled by alternative splicing

A proportion of *H-ras* pre-mRNA is spliced to incorporate an alternative exon, termed IDX (intron D exon), which contains an in-frame translational termination codon that prevents expression of the genetic information specified by the exon IV as shown in **Fig. 1** (Cohen et al, 1989). The abundance of these transcripts is low, apparently due to message instability or defective

processing. The predicted product of the alternate transcript (p19) lacks transforming potential, since the C terminal sequence of p21 that is necessary for attachment of the protein to the inner site of the cellular membrane is absent. It is suggested that alternative splicing patterns operate to suppress the *H-ras* p21 expression. This negative control is abolished by mutations that interfere with this process.

VII. Overexpression of *ras* genes in human tumors

Overexpression of *ras* genes is a common event in human tumors (reviewed by Zachos and Spandidos, 1997). **Table I** summarizes the experimental results by indicating the tumor type where elevated expression of *ras* genes was observed, the frequency of the overexpression, the activated member of the *ras* gene family, the stage in tumorigenesis and correlation of altered *ras* gene expression with prognosis of the disease. Where no particular *ras* gene is mentioned (referred as: *ras*), no discrimination between the *ras* family members was performed, nor was their status (mutated or wild-type alleles) defined.

Elevated *ras* gene expression was observed in human neuroblastomas (Spandidos et al, 1992), head and neck tumors (Field, 1991), esophageal (Abdelatif et al, 1991), laryngeal (Kiaris et al, 1995), thyroid (Papadimitriou et al, 1988), lung (Miyamoto et al, 1991), liver (Tiniakos et al, 1989), small intestine (Spandidos et al, 1993), stomach (Motojima et al, 1994), pancreatic (Song et al, 1996), colorectal (Spandidos and Kerr, 1994), breast (Dati et al, 1991), bladder (Ting-jie et al, 1991), endometrial

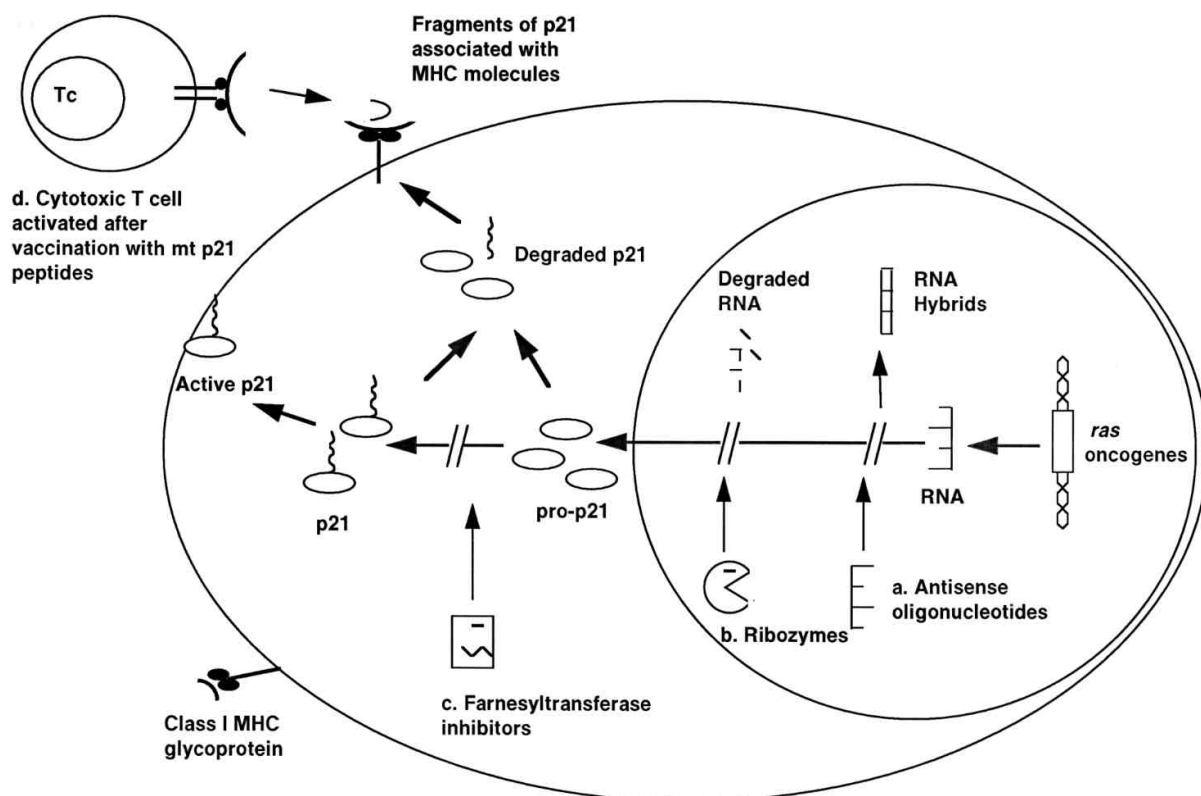


Figure 2. A synopsis of molecular therapeutic strategies developed against activated *ras* oncogenes. Strategies include antisense oligonucleotides, ribozymes, farnesyltransferase inhibitors and activation of T-cell response after mutant Ras peptide vaccination of patients.

(Long et al, 1988) and ovarian tumors (Scambia et al, 1993) and in leukemias (Gougopoulou et al, 1996). The frequency of elevated expression of the *ras* family of genes varies from 30% in endometrial and colorectal tumors, to 85-90% of cases in endometrial, lung and laryngeal tumors. In a number of tumors including neuroblastomas, head and neck, thyroid, colorectal and bladder cancers, *ras* overexpression is considered to be an early genetic event. However, in lung, stomach, endometrial and ovarian lesions, overexpression of *ras* genes appears in a later stage in tumorigenesis. Elevated expression of the Ras p21 protein is correlated with poor prognosis in lung, stomach, colorectal, bladder and ovarian lesions, whereas it is a favourable marker for neuroblastomas and head and neck tumors.

VIII. Molecular therapeutic strategies

The development of effective molecular strategies for therapy is the aim of tumor biology. Current therapeutic strategies include ribozymes against mutant *ras* gene products, antisense strategies, inhibitors of Ras protein post-translational modifications and Ras peptide vaccination (Fig. 2).

A. Ribozymes

Molecular biology applies the site-specific RNase properties of ribozymes to gene therapy for cancer. The anti-*ras* ribozymes are designed to cleave only activated *ras* RNA (Fig. 2). To develop this strategy into practical means, methods must be developed to accomplish high efficiency delivery of the ribozyme to target neoplastic tissue. An adenoviral-mediated delivery was designed (Feng et al, 1995). Using anti-Ras ribozymes, it was possible to reverse the neoplastic phenotype in mutant H-*ras* expressing tumor cells with high efficiency (Kashani-Sabet et al, 1994).

B. Antisense strategies

The antisense strategy involves reduction of a particular gene expression by introduction of a cDNA segment in antisense orientation, in order to bind the target mRNA and prevent its translation (Stein and Cheng, 1993) (Fig. 2). Critical to the success of such an antisense agent is its ability to enter living cells, to specifically bind the target mRNA and induce RNase-H cleavage of the target RNA. Activated *ras* genes, by mutation or overexpression, are a common target of these

therapeutic trials in cell-free and *in vitro* systems (Monia et al, 1992, Schwab et al, 1994).

C. Inhibitors of Ras post-translational modifications

Farnesylation of the CAAX motif of Ras protein is essential for the subcellular localization of Ras to the plasma membrane and is critical to Ras cell-transforming activity. Inhibitors of farnesyltransferase have been developed as potential cancer therapeutic agents (Gibbs et al, 1994) (**Fig. 2**). Requirements for Ras farnesylation inhibitors include specificity for farnesyl protein transferase, ability to inhibit post-translational modifications of the mutant *ras* specifically, high potency, activity *in vivo* and lack of toxicity (Kelloff et al, 1997).

D. Ras peptide vaccination

Ras peptide vaccination is a recent, developing molecular strategy for cancer therapy. Mutant Ras peptides are candidate vaccines for specific immunotherapy in cancer patients. An amount of mutant Ras p21 is degraded in the cytoplasm and fragments are attached with class I MHC glycoproteins, in the outer surface of the cell membrane (**Fig. 2**). When vaccinated with a synthetic Ras peptide representing the *ras* mutation in tumor cells, a transient Ras-specific T-cell response is induced, towards the fragments of mutant Ras protein associated with MHC molecules. Ras peptide vaccination was proved to be effective in 40% of patients with pancreatic cancer (Gjertsen et al, 1995, 1996). However, peptide vaccination of patients, like all other gene therapy strategies previously mentioned, requires considerable development before useful anti-cancer agents can emerge.

IX. Concluding remarks

Regulation of the *c-H-ras1* gene expression is a complicated procedure, including regulation by a variety of regulatory proteins (transcription factors, hormone receptors, tumor-suppressor proteins), alternative mechanisms (methylation, splicing) and by sequences located in the promoter region, in introns and downstream of the coding sequence. Understanding the molecular mechanisms of the expression of *ras* genes is of great significance for studying human tumorigenic events and developing effective strategies for gene therapy.

References

- Abdelatif OMA, Chandler FW, Mills LR, McGuire BS, Pantazis CG and Barret JM (1991) Differential expression of *c-myc* and *H-ras* oncogenes in Barret's epithelium-a study using colorimetric *in situ* hybridization. **Arch Pathol Lab Med** 115, 880-885.
- Avruch J, Zhang X and Kyriakis JM (1994) Raf meets Ras: completing the framework of a signal transduction pathway. **TIBS** 19, 279-283.
- Barak Y, Gottlieb E, Juven-Gershon T and Oren M (1994) Regulation of *mdm2* expression by p53: alternative promoters produce transcripts with nonidentical translation potential. **Genes Dev** 8, 1739-1749.
- Barbacid M (1987) *ras* genes. **Ann Rev Biochem** 56, 779-827.
- Beato M, Herrlich P and Schutz G (1995) Steroid hormone receptors: many actors in search of a plot. **Cell** 83, 851-857.
- Beato M (1989) Gene regulation by steroid hormones. **Cell** 56, 335-344.
- Berchuck A, Kohler MF, Marks JR, Wiseman R, Boyd J and Bast RC (1994) The p53 tumor suppressor gene is frequently altered in gynecologic cancers. **Am J Obstet Gynecol** 170, 246-252.
- Berg JM (1992) Sp1 and the subfamily of zinc finger proteins with guanine-rich binding sites. **Proc Natl Acad Sci USA** 89, 11109-11110.
- Bishop JM (1991) Molecular themes in oncogenesis. **Cell** 64, 235-248.
- Borello MG, Pierotti MA, Bongarzone I, Donghi R, Modellini P and Della Porta G (1987) DNA methylation affecting the transforming activity of the human *Ha-ras* oncogene. **Cancer Res** 47, 75-79.
- Boyes J and Bird A (1991) DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. **Cell** 64, 1123-1134.
- Boulikas T (1994) A compilation and classification of DNA binding sites for protein transcription factors from vertebrates. **Crit Rev Eukaryot Gene Exp** 4, 117-321.
- Cohen JB, Broz SD and Levinson AD (1989) Expression of the *H-ras* proto-oncogene is controlled by alternative splicing. **Cell** 58, 461-472.
- Cohen JB, Maureen VW and Levinson AD (1987) A repetitive sequence element 3' of the human *c-Ha-ras1* gene has enhancer activity. **J Cell Physiol (Suppl)** 5: 75-81.
- Comb M and Goodman HM (1990) CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. **Nucleic Acids Res** 18, 3975-3982.
- Dati C, Muraca R, Tazartes O, Antoniotti S, Perrotean I, Giai M, Cortese P, Sismondi P, Saglio G and DeBortoli M (1991) *c-ErbB-2* and Ras expression levels in breast cancer are correlated and show a cooperative association with unfavorable clinical outcome. **Int J Cancer** 47: 833-838.
- Dittmer D, Pati S, Zambetti G, Chu S, Teresky AK, Moore M, Finlay C and Levine AJ (1993) Gain of function mutations in p53. **Nat Genet** 4, 42-46.
- Easton D, Ford D and Bishop D (1995) Breast and ovarian cancer incidence in BRCA1 mutation carriers. **Am J Hum Genet** 56, 265-271.

- Feinberg AP and Vogelstein B (1983) Hypomethylation of *ras* oncogenes in primary human cancers. **Biochem Biophys Res Commun** 198, 47-54.
- Feng M, Cabrera G, Deshane J, Scanlon KJ and Curiel DT (1995) Neoplastic reversion accomplished by high efficiency adenoviral-mediated delivery of an anti-*ras* ribozyme. **Cancer Res** 55, 2024-2028.
- Field JK (1991) Prognostic implications of *ras* oncogene expression in head and neck squamous cell carcinoma. In: DA Spandidos, ed. The superfamily of *ras* related genes: **New York, Plenum Press**, 213-226.
- Gibbs JB, Oliff A and Kohl NE (1994) Farnesyltransferase inhibitors: Ras research yields a potential cancer therapeutic. **Cell** 77, 175-178.
- Gjertsen MK, Bakka A, Breivik J, Saeterdal I, Gadde-Dahl T 3rd, Stokke KT, Salheim BG, Egge TS, Soreide O, Thorsby E and Gaudernack G (1996) *Ex vivo* *ras* peptide vaccination in patients with advanced pancreatic cancer: results of a phase I/II study. **Int J Cancer** 65, 450-453.
- Gjertsen MK, Bakka A, Breivik J, Saeterdal I, Solheim BG, Soreide O, Thorsby E and Gaudernack G (1995) Vaccination with mutant *ras* peptides and induction of T-cell responsiveness in pancreatic carcinoma patients carrying the corresponding RAS mutation. **Lancet** 346, 1399-1400.
- Gougopoulou DM, Kiaris H, Ergazaki M, Anagnostopoulos NI, Grigoraki Y and Spandidos DA (1996) Mutations and expression of the *ras* family genes in leukemias. **Stem Cells** 14, 725-729.
- Hainaut P (1995) The tumor suppressor protein p53: a receptor to genotoxic stress that controls cell growth and survival. **Curr Opin Oncol** 7, 76-82.
- Hashimoto-Gotoh T, Kikuno R, Takahashi M and Honkawa H (1988) Possible role of the first intron of c-H-*ras* in gene expression: anti-cancer elements in oncogenes. **Anticancer Res** 8, 851-860.
- Hashimoto-Gotoh T, Takahashi M, Kikuno R, Ishihara H and Tezuka K (1992) Unusual sequence conservation in intron-1 of c-H-*ras* oncogene and its effect on p21 protein synthesis. In: Spandidos DA, ed. Current Perspectives of Molecular Cellular Oncology. London: **JAI Press**, 211-230.
- Honkawa H, Masahashi W, Hashimoto S, and Hashimoto-Gotoh T (1987) Identification of the principal promoter sequence of the c-Ha-*ras* transforming oncogene: deletion analysis of the 5' flanking region by focus formation assay. **Mol Cell Biol** 7, 2933-2940.
- Hyder SM, Stancel GM and Loose-Mitchell DS (1994) Steroid hormone-induced expression of oncogene encoded nuclear proteins. **Crit Rev Eucaryot Gene Expr** 4, 55-116.
- Ishii S, Kadonaga JT, Tjian R, Brandy JN, Merlino GT and Pastan I (1986) Binding of the Sp1 transcription factor by the human Harvey *ras1* proto-oncogene promoter. **Science** 232, 1410-1413.
- Ishii S, Merlino GT and Pastan I (1985) Promoter region of the human Harvey *ras* proto-oncogene: Similarity to the EGF receptor proto-oncogene promoter. **Science** 230, 1378-1381.
- Jones PA (1996) DNA methylation errors and cancer. **Cancer Res** 56, 2463-2466.
- Kashani-Sabet M, Funato T, Florenes VA, Fostad O and Scanlon KJ (1994) Suppression of the neoplastic phenotype *in vivo* by an anti-*ras* ribozyme. **Cancer Res** 54, 900-902.
- Katsaros D, Theillet C, Zola P, Iouason G, Sanfilippo B, Isaia E, Arisio R, Giardina G and Sismondi P (1995) Concurrent abnormal expression of *erbB-2*, *myc* and *ras* genes is associated with poor outcome of ovarian cancer patients. **Anticancer Res** 15, 1501-1510.
- Kelloff GJ, Lubet RA, Fay JR, Steele VE, Boone CW, Crowell JA and Sigman CC (1997) Farnesyl protein transferase inhibitors as potential cancer chemopreventives. **Cancer Epidemiol Biomarkers Prev** 6, 267-282.
- Kiaris H and Spandidos DA (1995) Analysis of H-*ras*, K-*ras* and N-*ras* genes for expression, mutation and amplification in laryngeal tumours. **Int J Oncol** 7, 75-80.
- Kiaris H and Spandidos DA (1995) Mutations of *ras* genes in human tumors (Review). **Int J Oncol** 7, 413-421.
- Kihana T, Hamada K, Inoue Y, Yano N, Iketani H, Murao S, Ukita M and Matsuura S (1995) Mutation and allelic loss of the p53 gene in endometrial carcinoma. **Cancer** 76, 72-78.
- Krontiris TG, Devlin B, Karp DD, Robert NJ and Risch N (1993) An association between the risk of cancer and mutations in the HRAS 1 minisatellite locus. **N Eng J Med** 329, 517-523.
- Kumar R, Sukumar S and Barbacid M (1990) Activation of *ras* oncogenes preceding the onset of neoplasia. **Science** 248, 1101-1104.
- Lee W and Keller EB (1991) Regulatory elements mediating transcription of the human Ha-*ras* gene. **J Mol Biol** 220, 599-611.
- Levesque MA, Katsaros D, Yu H, Zola P, Sismondi P, Giardina G and Diamantis EP (1995) Mutant p53 overexpression is associated with poor outcome in patients with well or moderately differentiated ovarian carcinoma. **Cancer** 75, 1327-1338.
- Lewin B (1991) Oncogenic conversion by regulatory changes in transcription factors. **Cell** 64, 303-312.
- Long CA, O'Brien TJ, Sanders MM, Bard DS and Quirk JG Jr (1988) *ras* oncogene is expressed in adenocarcinoma of the endometrium. **Am J Obstet Gynecol** 159: 1512-1516.
- Lowndes NF, Paul J, Wu J and Allan M (1989) c-Ha-*ras* gene bidirectional promoter expressed *in vitro*. **Mol Cell Biol** 9: 3758-3770.
- Lowy DR and Willumsen BM (1993) Function and regulation of *ras*. **Annu Rev Biochem** 62, 851-891.
- Macara IG, Lounsbury KM, Richards SA, McKiernan C and Bar-Sagi D (1996) The Ras superfamily of GTPases. **FASEB J** 10, 625-630.
- Marshall MS (1995) Ras target proteins in eucaryotic cells. **FASEB J** 9, 1311-1318.
- McGrath JP, Capon DJ, Smith DH, Chen EY, Seeburg PH, Goeddel PV and Levinson AD (1983) Structure and

- organization of the human *Ki-ras* proto-oncogene and a related processed pseudogene. **Nature** 304, 501-506.
- Mermod N, O'Neill EA, Kelly TJ and Tjian R (1989) The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. **Cell** 58, 741-753.
- Miyamoto H, Harada M, Isobe H, Akita HD, Haneda H, Yamaguchi E, Kuzumaki N and Kawakami Y (1991) Prognostic value of nuclear DNA content and expression of the *ras* oncogene product in lung cancer. **Cancer Res** 51, 6346-6350.
- Monia BP, Johnston JF, Eckers DJ, Zounes MA, Lima WF and Freier SM (1992) Selective inhibition of mutant *Ha-ras* mRNA expression by antisense oligonucleotides. **J Biol Chem** 267, 19954-19962.
- Motojima K, Furui J, Kohara N, Izawa K, Kanematsu T and Shiku H (1994) Expression of Kirsten-*ras* p21 in gastric-cancer correlates with tumor progression and is prognostic. **Diagn Mol Pathol** 3, 184-191.
- Nagase T, Ueno Y and Ishii S (1990) Transcriptional control of the human Harvey *ras* proto-oncogene: role of multiple elements in the promoter region. **Gene** 94, 249-253.
- Papadimitriou K, Yiagnis M, Tolis G and Spandidos DA (1988) Immunohistochemical analysis of the *ras* oncogene protein in human thyroid neoplasms. **Anticancer Res** 8, 1223-1228.
- Phelan CM, Rebbeck TR, Weber BL, Devilee P, Rutledge MH, Lynch HT, Lenoir GM, Stratton MR, Easton DF, Ponder BA, Cannon-Albright C, Larsson C, Goldgar DE and Narod SA (1996) Ovarian cancer risk in *BRCA1* carriers is modified by the *HRAS1* variable number of tandem repeat (VNTR) locus. **Nat Genet** 12, 309-311.
- Pintzas A and Spandidos DA (1991) Sp1 specific binding sites within the human *H-ras* promoter: potential role of the 6 bp deletion sequence in the T24 *H-ras1* gene. **Anticancer Res** 11: 2067-2070.
- Pulciani S, Santos E, Long LK, Sorrentino V and Barbacid M (1985) *ras* gene amplification and malignant transformation. **Mol Cell Biol** 5, 2836-2841.
- Rachal MJ, Yoo H, Becker FF and Lapeyre J-N (1989) In vitro DNA cytosine methylation of cis-regulatory elements modulates c-*Ha-ras* promoter activity in vivo. **Nucleic Acids Res** 17, 5135-5147.
- Scambia G, Catozzi L, Panici PB, Ferrandina G, Coronetta F, Barazzi R, Baiocchi G, Uccelli L, Piffanelli A and Mancuso S (1993) Expression of *ras* oncogene p21 protein in normal and neoplastic ovarian tissues: correlation with histopathologic features and receptors for estrogen, progesterone and epidermal growth factor. **Am J Obstet Gynecol** 168, 71-78.
- Schwab G, Chavany C, Duroux I, Goubin G, Lebeau J, Helene C and Saison-Bechmoaras T (1994) Antisense oligonucleotides adsorbed to polyalkylcyanoacrylate nanoparticles specifically inhibit mutated *Ha-ras*-mediated cell proliferation and tumorigenicity in nude mice. **Proc Natl Acad Sci USA** 91, 10460-10464.
- Song MM, Nio Y, Sato Y, Tamura K and Furuse K (1996) Clinicopathological significance of *Ki-ras* point mutation and p21 expression in benign and malignant exocrine tumors of the human pancreas. **Int J Pancreatol** 20, 85-93.
- Spandidos DA, Arvanitis D and Field JK (1992) *Ras* p21 expression in neuroblastomas and ganglio-neuroblastomas: correlation with patients' prognosis. **Int J Oncol** 1, 53-58.
- Spandidos DA, Zoumpourlis V, Zachos G, Toas SH and Halazonetis TD (1995) Specific recognition of a transcriptional element within the human *H-ras* proto-oncogene by the p53 tumor suppressor. **Int J Oncol** 7, 1029-1034.
- Spandidos DA and Holmes L (1987) Transcriptional enhancer activity in the variable tandem repeat DNA sequence downstream of the human *Ha-ras1* gene. **FEBS Lett** 218, 41-46.
- Spandidos DA and Kerr IB (1994) Elevated expression of the human *ras* oncogene family in premalignant and malignant tumours of the colorectum. **Br J Cancer** 49, 681-688.
- Spandidos DA, Liloglou T, Arvanitis D and Gourtsoyiannis NC (1993) *ras* gene activation in human small intestinal tumors. **Int J Oncol** 2, 513-518.
- Spandidos DA, Nichols RAB, Wilkie NM and Pintzas A (1988) Phorbol ester-responsive *H-ras1* gene promoter contains multiple TPA-inducible/AP-1-binding consensus sequence elements. **FEBS Lett** 240, 191-195.
- Spandidos DA and Pintzas A (1988) Differential potency and trans-activation of normal and mutant T24 human *H-ras1* gene promoters. **FEBS Lett** 232, 269-274.
- Spandidos DA (1991) The superfamily of *ras*-related genes. New York: **Plenum Press**, 1-338.
- Stein CA and Cheng YC (1993) Antisense oligonucleotides as therapeutic agents - is the bullet really magical? **Science** 261, 1004-1012.
- Ting-jie M, Ze W and Nianli S (1991) Correlation between the expression of the p21 *ras* oncogene product and the biological behavior of bladder tumors. **Eur Urol** 20, 307-310.
- Tiniakos D, Spandidos DA, Kakkanas A, Pintzas A, Pollice L and Tiniakos G (1989) Expression of *ras* and *myc* oncogenes in human hepatocellular carcinoma and non-neoplastic liver tissues. **Anticancer Res** 9, 715-722.
- Trepicchio WL and Krontiris TG (1992) Members of the *rel*/NF- κ B family of transcriptional regulatory proteins bind the *HRAS1* minisatellite DNA sequence. **Nucleic Acids Res** 20, 2427-2434.
- Trimble WS and Hozumi N (1987) Deletion analysis of the c-*Ha-ras* oncogene promoter. **FEBS Lett** 219, 70-74.
- Yang-Yen H-F, Chambard J-C, Sun Y-L, Smeal T, Schmidt TJ, Drouin J and Karin M (1990) Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. **Cell** 62, 1205-1215.
- Yates J, Warren N, Reisman D and Sugden B (1984) A cis-acting element from the Epstein-Barr viral genome that permits stable replication at recombinant plasmids in latently infected cells. **Proc Natl Acad Sci USA** 81, 3806-3810.
- Waterman JLF, Shenk JL and Halazonetis TD (1995) The dihedral symmetry of the p53 tetramerization domain

- mandates a conformational switch upon DNA binding. **EMBO J** 14, 512-519.
- Westaway D, Parkoff J, Moscovici C and Varmus HE (1986) Identification of a provirally activated c-Ha-ras oncogene in an avian nephroblastoma via a novel procedure: cDNA cloning of a chimaeric viral-host transcript. **EMBO J** 5, 301-309.
- Willumsen BM and Christensen A (1984) The p21 *ras* C-terminus is required for transformation and membrane association. **Nature** 310, 583-586.
- Wu X, Bayle JH, Olson D and Levine AJ (1993) The p53-*mdm2* autoregulatory feedback loop. **Genes Dev** 7, 1126-1132.
- Zachos G and Spandidos DA (1997) Expression of *ras* proto-oncogenes: regulation and implications in the development of human tumors. **Crit Rev Oncol Hematol** 26, 65-75.
- Zachos G, Zoumpourlis V, Sekeris CE and Spandidos DA (1995) Binding of the glucocorticoid and estrogen receptors to the human H-*ras* oncogene sequences. **Int J Oncol** 6, 595-600.
- Zachos G, Varras M, Koffa M, Ergazaki M and Spandidos DA (1996a) The association of the H-*ras* oncogene and steroid hormone receptors in gynecological cancer. **J Exp Ther Oncol** 1, 335-341.
- Zachos G, Varras M, Koffa M, Ergazaki M and Spandidos DA (1996b) Glucocorticoid and estrogen receptors have elevated activity in human endometrial and ovarian tumors as compared to the adjacent normal tissues and recognize sequence elements of the H-*ras* proto-oncogene. **Jpn J Cancer Res** 87, 916-922.
- Zambetti GP and Levine AJ (1993) A comparison of the biological activities of wild-type and mutant p53. **FASEB J** 7, 855-865.
- Zoumpourlis V, Zachos G, Halazonetis TD, Ergazaki M and Spandidos DA (1995) Binding of wild-type and mutant forms of P53 protein from human tumors to a specific DNA sequence of the first intron of the H-*ras* oncogene. **Int J Oncol** 7, 1035-1041.