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Apoptosis Pathways in Ovarian Cancer

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1. Introduction

Tumour initiation and progression are driven by constitutively activated oncogenes mediating deregulation of the balance between cell death- and survival pathways. Among the most relevant signalling cascades activated in the majority of tumour types, the RAS/mitogen-activated protein kinase (Ras/MAPK), the phosphatidyl inositol-3-kinase/protein kinase B (PI3K/PKB) and the protein kinases C (PKC) signalling cascades were postulated (Weinstein, 1987; Nicosia et al., 2003; Roberts and Der, 2007; McCubrey et al., 2007; Breitkreutz et al., 2007). These cascades define individual characteristics of particular tumours and consequently their individual responsiveness to cancer therapy.

In this chapter, we will address the characteristics of the apoptotic signalling pathways in ovarian carcinomas. Particular attention will be given to the *HRS* family of tumour suppressor genes encoding proteins with phospholipase activity and suppressed in the majority of ovarian malignancies. We will describe signalling cascades down regulating two well-characterized members of this family H-REV107-1/HRLS3/PLA2G16 and TIG3/RARRES/RIG1 in tumour cells. Furthermore, potential therapeutic consequences of the re-expression of these genes defined as a class II tumour suppressors will be discussed.

2. The HRS class II tumour suppressors are important mediators of IFN-γ and retinoid-dependent growth suppression and cell death in ovarian cancer

The *H-REV107*-related genes (*TIG3, H-REV107-1, HRSL2*) are known as inhibitors of proliferation of tumour cells in vivo and in vitro. While being almost ubiquitously expressed in normal tissues, down-regulation or complete loss of these genes in tumours and tumour cell lines have been reported. Expression can be reconstituted by different anti-proliferative signals such as interferons and retinoids, as well as by the inhibition of oncogenic pathways and interference with DNA methylation (Alessi et al., 1994; Husmann et al., 1998; Akiyama et al., 1999; Siegrist et al., 2001; Ito et al., 2001; Roder et al., 2002; Huang et al., 2002; Higuchi et al., 2003; Duvic et al., 2003). Re-activation of the H-REV107-1-related proteins and over-expression of the genes induce apoptosis or differentiation of tumour cells.

2.1 HRS family members encode LRAT-related phospholipid-metabolizing enzymes

Two independent groups (Hughes and Stanway, 2000; Anantharaman and Aravind, 2003) have unfolded the phylogenetic relationship between *H-REV107-1*-related genes, LRAT (Lecithin retinol acyltransferase) and viral and bacterial peptidases in previous works.

Here we aimed to identify and describe H-REV107-1 homologs in different organisms in order to follow their origin and development during the evolution.

For that purpose, we performed an *in silico* analysis, using PSI-Blast and Blast-p screening in the NCBI non-redundant database. This analysis revealed 62 homologous proteins in eukaryotic and prokaryotic organisms. To identify phylogenetic relationships Tree Puzzle (Strimmer and von Haeseler, 1997) was applied (Fig. 1). The analysis revealed five closely related proteins, suggesting their origin from the same ancestor protein during the evolution. These proteins, including H-REV107-1 comprise a new protein family, which we designated here as the HRS (H-REV107-1-related proteins) protein family (Table 1).

The novel HRS family is composed of tumour suppressors, which negatively regulate cell survival, control signal transduction and induce differentiation.

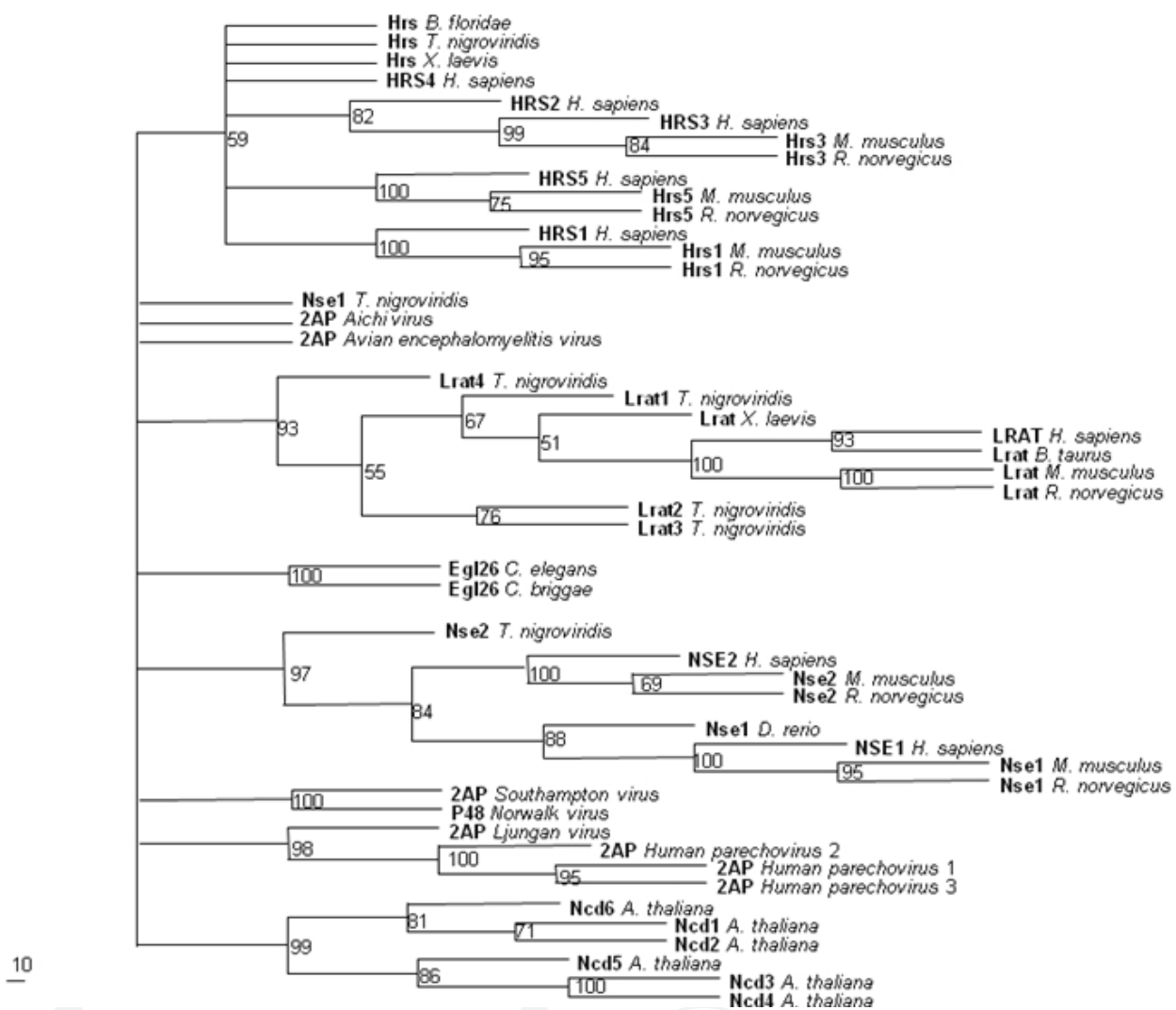| Suggested nomenklature	NCBI Synonyms	Chromosomal localization	Species	Publication - gene cloning	Acc. No gene	Acc. No protein	Function
HRS1	HRASLS	3q29 (194440-1944470 K)	Human	Ito et al. Cytogenet. Cell Genet. 93:36-39 (2001)	NM_020386	NP_065119	Unknown
Hrs1	A-C1, Hrasls, 2810012B06Rik	16	Mouse	Akiyama et al. J. Biol. Chem. 274: 32192-32197 (1999)	NM_013751	NP_038779	Inhibits growth of HRAS-transformed cells
Hrs1	similar to Hrasls		Rat	Uyama et al., Biochim.Biophys.Acta (2009)	XM_213590	XP_213590	PE N-acyltransferase lysophospholipid O-acyltransferase
HRS2	HRASLS2	11q12.3 (63076-63088 K)	Human		NM_017878	NP_060348	
HRS3	HRASLS3, H-REV107-1, H-REV107-3, hHrev-107, PLA2G16	11q12.3-q13.1 (63099-63138 K)	Human	Husmann et al. Oncogene 17: 1305-1312 (1998)	NM_007069	NP_009000	Phospholipase 2A, Induces apoptosis in ovarian cancer cells
Hrs3	Hrasls3	19	Mouse	Roder et al. J. Biol. Chem. 277: 30543-30550 (2002); Jaworski et al., Nat Med. 2009	NM_139269	NP_644675	major adipocyte phosphoslipase
Hrs3	Hrasls3, H-Rev107	1q	Rat	Hajnal et al. Oncogene 9: 479-490 (1994)	NM_017060	NP_056756	Inhibits growth of Ras-transformed cells in vivo and in vitro
HRS4	RARRES3, TIG3, RIG1, H-REV107-2	11q13.2 (63061-63070 K)	Human	DiSepio et al. PNAS 95: 14811-14815 (1998)	NM_004585	NP_004576	phospholipid methabolizing enzyme, Induces apoptosis in ovarian cancer cells
HRS5	HRLP5; iNAT	11q13.2 (62988-63015 K)	Human	Jin et al., Biochim.Biophys.Acta (2009); Jin et al., J.Biol. Chem (2007)	NM_054108	NP_473449	Ca-independent phosphatidylethanolamine N-acyltransferase
Hrs5	Hrlp5		Mouse		NM_025731	NP_080007	
Hrs5	Hrlp5		Rat		XM219546	XP_219546	
Hrs	unnamed		Fish		CAG09755	CAAE01015008	
Hrs	MGC68773		Frog		BCO60489	AAH60489	
Hrs	unnamed		Amphioxus		AF391288	AAM18866	

Table 1. Members of the HRS family

Fig. 1. Phylogeny of HRS and the related LRAT, NSE and NCD protein families
A maximum parsimony tree was generated with the help of Tree-Puzzle (Strimmer and von Haeseler 1997) and includes the eukaryotic members of the HRS, LRAT, NSE families, plant NC proteins, the *C.elegans* Egl26 proteins, and the viral 2A proteins.
First, only HRS proteins were aligned using the ClustlW algorithm (EMBL-EBI), then LRAT and NSE families. From the *C.elegans*, the viral and the plant proteins only regions with a high similarity to the HRS proteins were compared. Additional upstream and downstream motifs were cut out. As a result, all sequences had a comparable length of about 160 amino acid residues that corresponds the average length of the HRS proteins.
For the calculation of the phylogenetic relationships 1000 replicates were run. Branch support values are indicated at the nodes, distances are proportional to relative sequence divergence.

2.2 Clustered chromosomal localization of 4 human HRS genes on 11q13

Four of the five members of the human HRS family, *HRLS2*, *H-REV107-1*, *TIG3* and *HRLS5*, are localized in one cluster on chromosome 11q13, supporting the hypothesis of their origin from the same ancestor (Fig. 3). The *HRLS2* and *H-REV107-1* genes, encoding most closely related family members (Fig. 1A), are located next to each other. The *H-REV107-1* gene spans between 63099K and 63138K on the chromosome 11q, and directly downstream of it, from 63077K to 63088K, the *HRLS2* gene is located. The *TIG3* gene is positioned on the opposite DNA strand directly downstream of *HRLS2*, the gene has a small non-coding region and spans between 63070K and 63079K. The *HRLS5* gene 62897K and 63015K is also located on chromosome 11q13, but separated by two genes encoding the thymosin-like 5 (TMSL5) and the lectin, galactoside-binding, soluble, 12 (galectin 12/LGAL12) proteins, from the other HRS genes.

Earlier findings suggested that chromosomal alterations resulting in *HRS* gene down-regulation or loss are rather rare events in human carcinomas. Nevertheless, structural changes on 11q13 have been described in numerous cases and only recently methods such as array CGH and next generation sequencing (NGS) have improved the analysis such that the involvement of individual genes can now be analysed. Therefore, it cannot be excluded that future investigations might unravel smaller deletions influencing one of the clustered *HRS* genes on 11q13 in distinct tumour types.

2.3 Domain structure and enzymatic activity of the HRS family members

Phylogenetic analysis of HRS and HRS-related proteins revealed a high conservation within the so called NlpC/P60 domain (Anantharaman and Aravind, 2003). This sequence was indentified in LRAT proteins (lecithin retinol acyltransferase) as being essential for all-trans-retinol metabolism.

To analyse domain structure of other members of the HRS family, Clustl W alignment was performed. Using this program, 14 members of the HRS protein family found in the NCBI database, were analysed (Fig. 2).

The HRS proteins contain non-homologous proline-rich motifs on their N-termini (red line on the top of the alignment). The core parts of HRS proteins are highly conserved and contain the NlpC/P60 and NC domains (Fig. 2, blue and green boxes, respectively). We predicted three β-strands within the Nlpc/P60 domain (Fig. 2, blue arrows). The first and the second strand contain the conserved GDL and HWXXY motifs; the VXXLAP motif comprises the third strand. The region downstream the third β-strand with two conserved serine residues is likely to have the structure of α-helix (Fig. 2, green cylinder). The large NC domain depicted in Fig. 2 with a green box, contains a KALVK conserved motif of unknown function, two short stretches DXXG and NKXD, which are similar to conserved regions of GTPases (Akiyama et al., 1999; Bourne, Sanders, and McCormick 1990) and the NCEHFV conserved motif, characteristic for conventional NC domains. At the C-terminus, HRS proteins harbour a hydrophobic C-terminal α-helix,described as a membrane-binding domain.

Recently, a crystal structure of the NlpC/P60 domain of H-REV107-1 has being resolved (Ren et al., 2010b). Within this domain, a phospholipase active site consisting of a Cys-His-His triad was identified. The residues H23 and C113 play a pivotal role for the H-REV107-1 enzymatic activity (Ren et al., 2010a). Meantime, the enzymatic activity of the H-REV107-1, TIG3, HRASLS2 and HRLP5 proteins has been characterized as $PLA_{1/2}$- type hydrolysis, supporting a role of the HRS proteins in lipid metabolism.
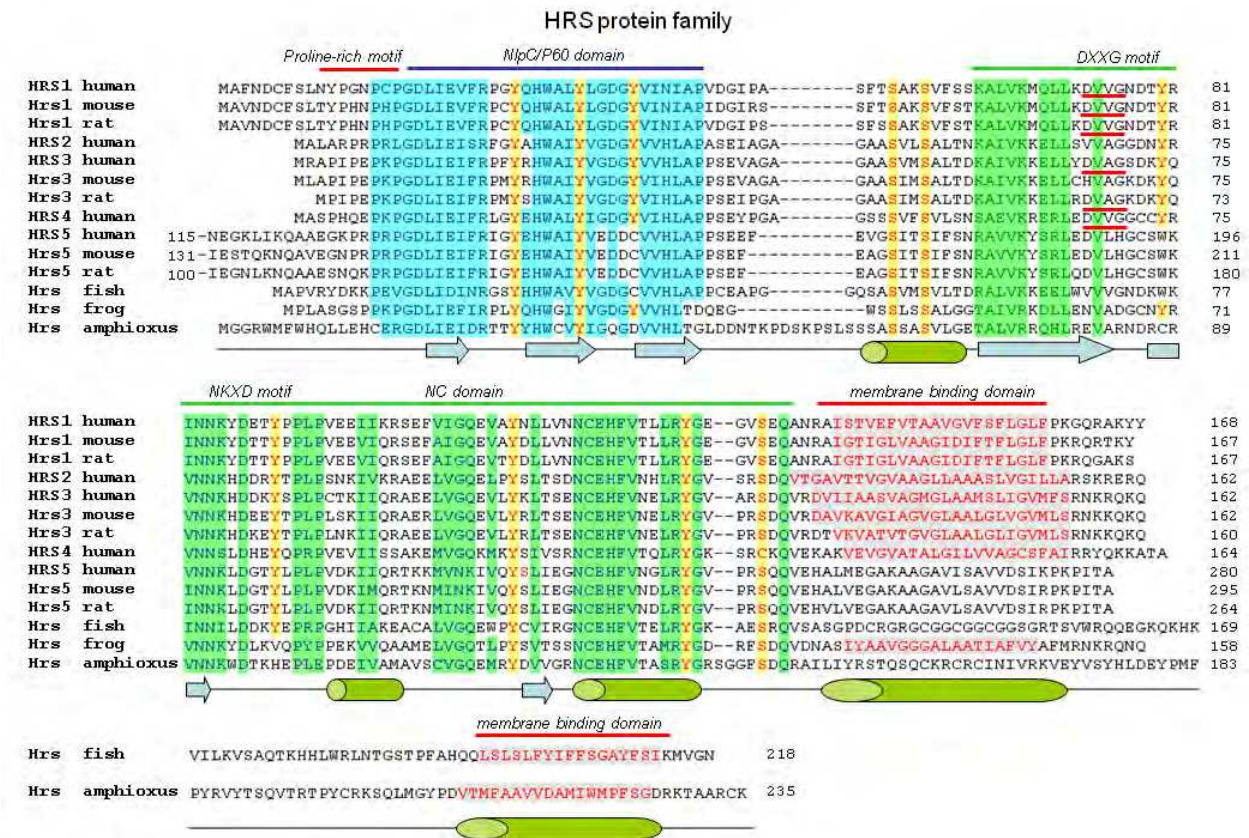
Fig. 2. HRS catalytic and protein-binding domains

Fourteen members of the HRS protein family found in the NCBI database were aligned using the Clustal W program as described in Figure 1B. Analysis, edition, and shading of conserved domains were performed with the help of the GenDoc freeware (http://www.psc.edu/biomed/genedoc/). The PSIPRED secondary structure prediction server was used to analyze potential secondary structures of the HRS protein sequences (http://bioinf.cs.ucl.ac.uk/psipred/) (McGuffin, Bryson, and Jones 2000).

The HRS proteins contain non-homologous proline-rich motifs on their N-termini (red line on the top of the alignment). The core parts of HRS proteins are highly conserved and contain the NlpC/P60 and NC domains (blue and green boxes, respectively). With a high prediction confidence of the PSIPRED standard analysis, three β-strands were defined within the Nlpc/P60 domain (blue arrows). The first and the second strand contain the conserved GDL and HWXXY motifs; the VXXLAP motif comprises the third strand. The region downstream the third β-strand with two conserved serine residues is likely to have a structure of α-helix (green cylinder). The large NC domain (green box) contains a KALVK conserved motif of unknown function, two short stretches DXXG and NKXD, which are similar to conserved regions of GTPases (Akiyama et al., 1999; Bourne, Sanders, and McCormick 1990) and the NCEHFV conserved motif, characteristic for conventional NC domains. At the C-terminus, HRS proteins harbour a hydrophobic C-terminal α-helix.
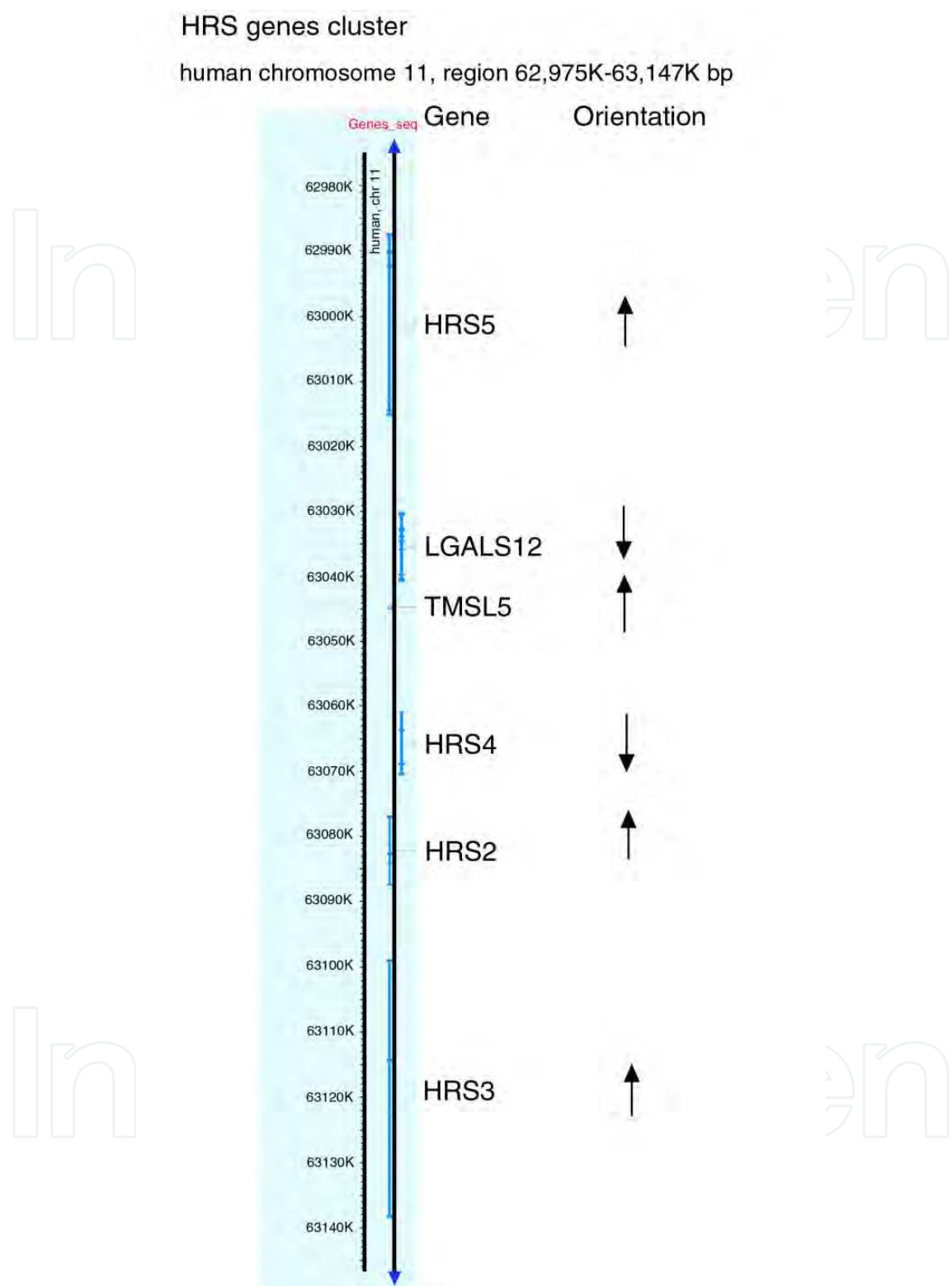
Fig. 3. *HRS2/HRLS2, HRS3/H-REV107-1/ HRS4/TIG3* and *HRS5/HRLS5* genes are localized on chromosome 11q13 in one cluster. Gene orientation, length and mapping of the chromosome regions are directly obtained from the NCBI Map View server.

Additionally to the NlpC/P60 domain, HRS proteins contain a proline-rich N-terminal domain, responsible for establishing protein-protein interactions and a variable

hydrophobic C-terminal α-helix, which directs and transiently binds the protein to intracellular membranes (Husmann et al., 1998; Nazarenko et al., 2007). Furthermore, a DXXG domain, also termed G3 motif, characteristic for RAS small GTPases, and mediating the binding of magnesium and γ-phosphate of GTP via the aspartic acid and glycine residue, respectively was identified (Kjeldgaard et al., 1996). However, a functional role of these motifs in HRS proteins has not been defined yet.

2.4 Reversible Inhibition of the HRS genes H-REV107-1 and TIG3 by oncogenic signalling cascades in tumours

Members of the HRS gene family *H-REV107-1* and *TIG3* belong to the so called class II tumour suppressors. The major characteristic of this class, postulated in 1997 by Ruth Sager, is their down-regulation in tumours via reversible mechanisms, however not through mutations or deletions (Sager, 1997). Once re-expressed, these genes can exhibit their tumour-suppressive function and thereby contribute to the inhibition of tumour progression.

2.4.1 IFNγ-mediated re-expression of H-REV107-1 leads to the induction of apoptosis in ovarian cancer cells

Rat *H-Rev107-1*, the founder gene of the HRS family, was cloned from a subtractive cDNA library (Hajnal et al., 1994). The rat *H-Rev107-1* gene, expressed in immortalized rat fibroblasts, was identified as a gene suppressed in an HRAS-transformed derivative, but re-expressed in a revertant cell line. Further experiments suggested that repression of *H-Rev107-1* in HRAS-transformed cells was functionally involved in HRAS-dependent transformation (Hajnal et al., 1994; Sers et al., 1997).

Repression of *H-Rev107-1* was also detected in KRAS-transformed rat ovarian epithelial cells suggesting that in contrast to other HRS genes, *H-Rev107-1* suppression in response to RAS oncogenes is not associated with the RAS isoform. Most interestingly, *H-Rev107-1* down-regulation upon KRAS-transformation appeared to be reversible and identified the *H-Rev107-1* gene as a target negatively regulated by the MEK-ERK pathway. The same observation was also made in PA1 human teratocarcinoma cells, which harbour an activated NRAS oncogene (Alessi et al., 1994) and suggested that *H-REV107-1* might be negatively affected by RAS oncogene-dependent signalling in general.

The human *H-REV107-1*, first described in 1998, was found ubiquitously expressed in normal human epithelial tissues (;Husmann et al., 1998). When compared to differentiated cells in situ, *H-REV107-1* is down-regulated in human tumour cell lines and tumour samples at the mRNA and at the protein level. Loss of *H-REV107-1* mRNA until now was detected in tumours derived from breast, lung, ovary, kidney and testis (Sers et al., 1997;Siegrist et al., 2001). In human ovarian carcinomas, we also demonstrated strongly diminished levels or complete loss of the H-REV107-1 protein. In ovarian carcinomas sequencing of the *H-REV107-1* coding region revealed no alterations within this region suggesting that *H-REV107-1* acts as class II tumour suppressor gene in these tissues.

A functional involvement of H-REV107-1 inactivation in ovarian tumours was demonstrated by the finding that reactivation of endogenous H-REV107-1 in H-REV107-1-negative ovarian carcinoma cells induces apoptosis. In these cells, loss of *H-REV107-1* expression can be reconstituted upon administration of Interferon gamma (IFNγ), a finding reported earlier from rat astrocytoma cells (Bartel, 2004). Up-regulation of *H-REV107-1* in response to IFNγ

works well at the mRNA level, yet only a small proportion of cells also express sufficient H-REV107-1 protein for detection. Most interestingly these cells undergo apoptosis (Sers et al., 2002). These observations made clear that H-REV107-1 is likely to interfere with the survival of ovarian cancer cells. Our work further supported this suggestion as we could show that H-REV107-1 is an inhibitor of PP2A whose function is required in ovarian carcinomas for cell survival (Nazarenko et al., 2007). This was the first hint indicating a role of the H-REV107-1 protein in the regulation of apoptotic intracellular signalling and will be discussed in part 3 of this chapter.

2.4.2 Mechanisms of H-REV107-1 suppression in ovarian carcinomas via anti-apoptotic pathways

The reversible down-regulation of *H-REV107-1* in ovarian cancer has prompted the investigation of the mechanisms responsible for suppression. The human *H-REV107-1* promoter is located directly upstream of a 408bp 5'UT sequence. The sequence harbours several potential transcription factor binding sites including an Interferon-responsive IRSE motif, a CREB site, potential AP-1 and c-REL binding sites (Fig. 4).

The IRSE site, a DNA-sequence bound by the Interferon regulatory factors IRF-1 and IRF-2, provides the structural basis for the observed induction of *H-REV107-1* upon administration of IFNγ and conditionally expressed IRF-1 (Alessi et al., 1994). Comparison of *IRF-1* and *H-REV107-1* levels between human ovarian carcinoma cells and immortalized human ovarian epithelial cells, revealed strongly diminished *IRF-1 and H-REV1017-1* levels in the tumour cell lines. This suggested that loss of *IRF-1* expression might be one of the mechanisms of *H-REV107-1* suppression in human ovarian carcinomas (Sers at al., 2002).

Surprisingly, there is no conservation between the human *H-REV107-1* and the mouse or rat *H-REV107-1* promoter region, suggesting a different regulation of human *H-REV107-1* and the rodent homologues. More importantly, it was shown that murine *H-REV107-1* can be regulated via DNA methylation. In view of human tumours, next steps will include addressing the question, whether this methylation-dependent suppression of *H-REV107-1* is a tumour-related process, or a developmental process during which tissue-specific expression profiles are established.

2.4.3 Physiological role of H-REV107-1 and its potential role in cancerogenesis

Meantime, the enzymatic function of H-REV107-1 has been defined (Ueda et al., 2009). The protein acts as a cytosolic Ca^{2+}-independent phospholipase Pla2G16, which catalyses esterolytic cleavage of glycerophospholipids to lysophospholipids. Supporting these data, a recent study in a knock-out model demonstrated that the H-rev107-1 physiological function is a major adipocyte phospholipase A_2 (AdPLA). The protein inhibited lipolysis in adipocytes, regulating adiposity on systemic level (Jaworski et al., 2009). Ablation of the H-rev107-1 led to a significantly higher rate of lipolysis, accompanied by an increase in cyclic AMP levels (Jaworski et al., 2009). The knock-out animals were resistant to high-fat feeding and leptin-deficiency mediated obesity. Albeit, a direct impact of the H-rev107-1 ablation on tumourigenesis in vivo has not been tested yet, the observed increase in lipolysis and elevated levels of cAMP, also common in tumour cells, suggest a potentially higher susceptibility of the H-rev107-1 knockout animals to tumour growth as compared to their wild type littermates.

Promoter sequence of the *H-REV107-1* gene

```
-997  TGAGACTGAGTTTCGCTTTGTTGCACAGGCTAGAGTGCAGTGGCGCGATC
                ISRE bzw.
              IRF-1/IRF-2 Element
-947  TTGGCTCACTGCAACCCCCACCTCCCGGGCTCAAGTGATTCTCCTGCCTC
-897  AGCCTCCCGAGTAGCTGGGACTACAGGCGCATGCCTCCACGCCCGGCTAA


-847  TTTTTTTGCATTTTTAGTAGAGACGGGGTTTCATCGTGTTAGCCAGCATGG
                                                   AP-4

-797  TCTCGATCTCTTGACCTCGTGATCTGCCCGCCTCGGCCTCCCAAAGTGCT
                AP-1

-747  GAGATTACAGGCGTGAGCCACCGCGCCCGGCCCTTGGTGGTATATTTTTA
-697  ACTCCTTCAGTTTTTAAACTATAAGCCCATTCTTGAGTGAAGGCGAAAGT


-647  AAACCCATCATGGCCCTGCAGTGTGATGTGTGTGCAGAGGTCGAGTGTGT
-597  GCGACTCCTGGATGCTGGGCGCGCAGGGCATGGGTGAGGCGGGAAGAGGC
-547  GGTGCCGGGGGCGCGGGCGTCCTGCAGTCGCCGGGCTCGGGACCGGGGCC

                                              GC-Box
-497  GGGCGCTCTGCGAGGCTCTCATTAGCCGGCGGCGCGGGGAGGGGCCGGGT

                          max. TSP ⁝▶
-447  GACCTCACGCCGGCCCGCCACCGCGGCCATTAGACCCGGTCCAATTGCTG

       CREB-1                        CCAAT-Box #1  CCAAT-Box #2

                                                       ········
-397  GGGCTGCAGCGCTGCCTCCGAGACCGCGAAGTGGGTGGATCGGGTCTTCC

       STAT-1                                          cRel #1
       ··········
-347  TGGAAGGTGCGATAAGGCCGGGCGAGGTGCCTGGGATGCTTCTCCCCTTC
            cRel #2

-297  CGCGAGGAAGAGATCTAATTGGGTAGGGCGGGTGTAGACTAGCCTGCCGA
-247  GCCGCCCGCTGGCACCTGCAGCCTCCTGGGCGCCCGCGGGCCCCGGCGAG
-197  AAAGTTGTTAAAGGGAGCGAGGTGGTTGTTCCTGGGGTACGAGGCGCGCC
-147  TCTCACGCCCTGCCCAACAGAAGCCGCAGTCCGTGGGGTCTGGAGACGCA
- 97  GTTTCCTTGTTAATGACAATAAATCCCTGCTCCCCCTGCCTCAGACATCT

                                                       +1
- 47  ACGCAGCGAAATCGAGCCTGGCCTTGAGGGTCCACACCGCGAGGAAGATG
+  4  CGTGCGCCCATT
```

Fig. 4. Promoter sequence of the human *H-REV107-1* gene. The translatioal start site is indicated by +1, 997 base pairs of upstream sequence are shown. Individual sequence motifs as identified by MatInspector are indicated.

The H-rev107-1 knockout model provides a first link between lipid metabolism and a tumour suppressive effect of phospholipases. Alterations in lipid metabolism, especially in phospholipids-related pathways and fatty acid biosynthesis are known to occur in ovarian carcinomas (Tania et al., 2010). . Thus, FAS (fatty acid synthase) is up-regulated in cancer cells and mediates activity of HER-2 (Gansler at al., 1997; Menendez et al., 2004). It has been suggested that HER-2 functions as a cellular energy sensor in response to the metabolic stress, supporting therapeutic advantages of combinatorial inhibition of HER-2 and FAS in HER-2-positive tumours. However, phospholipases PLA2 were known to function as positive regulators of cell proliferation and migration (Song et al., 2007), playing rather a tumour-promoting role. In contrast to that, we and other uncovered a tumour-suppressive function of H-REV107-1 and its related proteins functioning as PLA2 enzymes.

It is likely that these observations provide a new link between malignant transformation, tumour progression and alteration in lipid metabolism, which needs further investigations. An important aspect needs to be refurbished according to the latest findings, is a change of lipid metabolic in tumour-surrounding stroma. Recent data clearly demonstrate a key role of adipocytes in the preferential metastasis of ovarian cancer to omentum, indicating their function as an energy source for homing tumour cells (Nieman et al, 2011). These and other data support a significant role of metabolism regulation in tumours and tumour stroma, and suggesting that inclusion of metabolism-regulating agents in cancer therapy should be re-examined with respect to a potential pronounced beneficial effect on the efficacy of the treatment on a system level.

2.5 TIG3, a target of the MAPK signalling pathway, acts as a tumour suppressor in ovarian cancer cells

The *TIG3* gene was described independently by two groups (Husmann et al., 1998; DiSepio et al., 1998). DiSepio et al. had identified a close homologue of the rat *H-rev107-1* gene, named *RARRES/TIG3*, which was isolated from a differential display approach using Tazarotene-treated human keratinocytes. Tazarotene is a synthetic retinoid, developed for the treatment of psoriasis (Weinstein et al., 1997). Husmann et al. also described a gene closely related to the human *H-rev107-1*, named *H-REV107-2*, isolated during a sequencing project by Merck and the University of Washington. The H-REV107-2 protein differed from RARRES/TIG3 in a longer C-terminal region however; this was recently identified as an artefact (Lotz et al., 2005). Re-sequencing of the *H-REV107-2* cDNA construct revealed that the cDNA is identical to the *RARRES/TIG3* gene, referred further as *TIG3*. In addition, a similar sequence cloned from human gastric carcinoma cells was described as RIG1 (Huang et al., 2000). According to sequence comparisons, all proteins are identical except a difference of two amino acids between the proteins deduced from the TIG3 and the RIG1 sequence.

Expression analysis for *TIG3* performed on Multiple Tissue Northern Blots and Cancer Profiling Arrays suggested expression of the gene in normal ovary and in many other tissues. Similar to *H-REV107-1*, *TIG3* expression was down-regulated in human ovarian carcinomas and tumour-derived cell lines (DiSepio et al., 1998;Duvic et al., 2000;Shyu et al., 2003;Higuchi et al., 2003;Sturniolo et al., 2003;Lotz et al., 2005) and can be re-expressed upon treatment with IFNγ or retinoid and its analogous (Weinstein et al., 1997).

Up-regulation of *TIG3* by IFNγ occurs in the same cells in which also *H-REV107-1* can be induced by this cytokine. Within the 5´ regulatory sequence of the *TIG3* gene an IRF-responsive element is present 84 base pairs upstream of the translational start site. However, compared to the related *H–REV107-1* gene, *TIG3* mRNA levels after IFNγ-administration follow a different kinetics suggesting that during the IFNγ-dependent apoptosis, these genes are involved at different stages of the process.

Deregulation of retinoic acid receptors has been involved in ovarian tumours, indicating an essential role of genes targeted by retinoic acid signalling in the prevention of transformation (Benoit et al., 2001;Sun and Lotan, 2002). Furthermore, retinoids represent a promising alternative chemotherapeutic approach for the treatment of late stage ovarian cancer (Zhang et al., 2000;Fields et al., 2007) Consequently, TIG3, involved into retinoic signalling, is likely to be one of the potential mediators for a successful anti-cancer therapy of ovarian carcinomas.

In addition to the retinoic acid responsiveness, we recently detected a negative regulation of *TIG3* via an activated MEK-ERK signalling pathway and a positive regulation via IFN-γ in ovarian carcinoma cells (Lotz et al., 2005). Thus, like the related *H-REV107-1* gene, *TIG3* is a target of the oncogenic MEK-ERK signalling pathway. TIG3 itself can dampen the activity of ERK, which suggests an involvement of TIG3 in a negative feedback loop for the control of ERK activity. Inducible and constitutive overexpression of *TIG3* cDNA, resulted in growth suppression of A27/80 ovarian carcinoma cells indicating a functional role of the protein in cell growth control (DiSepio et al., 1998; Lotz et al., 2005). However, the mechanisms of ovarian cancer-specific MEK-ERK-dependent TIG3-suppression are unknown.

An important finding was reported by Ou et al., showing that TIG3 mediates IFN-γ dependent down-regulation of HER-2 via regulation of the PI3-kinase pathway (Ou et al., 2008). Using human ovarian carcinoma cell lines OVCAR-3, SKOV-3, and TOV-21G, the group demonstrated an increase of the *TIG3* mRNA levels within 2 hours upon administration of IFN-γ to the cells. Up-regulation of *TIG3* correlated with the down-regulation of p185 protein, which could be restored by the application of siRNA against *TIG3*. A promoter activity assays allowed to demonstrate that TIG3 acts in a HER-2 dependent manner, by a diminishment of the HER-2 activity. Abrogation of HER-2 signalling resulted in a down-regulation of the p185 subunit of the PI3-Kinase. Additionally, VEGF (vascular endothelial growth factor) secretion was regulated in a TIG3-HER-2 dependent manner in a model system. The anti-proliferative, HER-2-inhibiting effect of TIG3 could be abrogated by overexpression of HGR, a member of the neuregulin family activating epidermal growth factor receptor family members and restoring p185 expression (Ou et al., 2008).

This work shows that TIG3 is an important regulator of survival signalling in ovarian carcinomas. Further experiments are necessary, verifying the in vitro observations in animal models of ovarian cancer. Additionally, examination of human ovarian carcinomas and a correlative analysis of TIG3, HER-2 and p185 expression will allow determining the general relevance the observed phenomenon.. Furthermore, due to the co-regulation of TIG3 and H-REV107-1 via IFN and MAPK signalling, a reactivation of both genes for therapeutic purposes might exhibit an enhanced anti-apoptotic effect.

3. H-REV107-1/HRLS3-driven interplay between PP2A and PKC signal transduction pathways in ovarian carcinomas

In our previous work, we demonstrated that the class II tumour suppressor H-REV107-1 defined as an enzyme with a phospholipase activity (Jaworski et al., 2009) induces apoptosis in ovarian cancer cells by inhibition of a specific pool of serine/threonine phosphatase PP2A followed by the activation of the atypical PKCζ (Nazarenko et al., 2007;Nazarenko et al., 2010).

The PKC family comprises 3 groups of kinases that display very distinct modes of activation and function. The classical PKCs (α,β,γ) are activated in a calcium-dependent manner through phosphatidylserine (PS) and diacylglycerol (DAG). The novel PKCs (δ, ε, η, θ), are also regulated through PS and DAG, but are calcium-independent. Finally, there are the atypical PKCs (ζ, ι/λ) that require neither calcium nor DAG, but in some cases PS, for activation (Parker and Murray-Rust, 2004;Mackay and Twelves, 2007;Breitkreutz et al., 2007). The different PKC isoenzymes are involved in the regulation of cell survival in normal organs and during tumourigenesis (Shayesteh et al., 1999;Leitges et al., 2001;Martin et al., 2002;Parker and Murray-Rust, 2004;Yin et al., 2005;Moscat et al., 2006). Among the classical PKCs, loss of PKCα in ovarian carcinoma was found to be correlated with increased malignancy (Weichert et al., 2003). While the classical PKCα is down-regulated in ovarian carcinomas, the novel PKCθ and PKCε were found up-regulated in this tumour, yet no functional consequence has been inferred from this deregulation. In addition to the novel PKCθ and PKCε, also the atypical PKCι is highly expressed in ovarian carcinomas and acts as a cooperating oncogene with mutant RAS (Zhang et al., 2006).

Recently, we demonstrated that forced expression of H-REV107-1 in ovarian carcinoma cell lines resulted in the inhibition of PP2A activity, re-activation of PP2A target proteins, among them PKCζ, and induction of apoptosis (Nazarenko et al., 2007). Importantly, not only tumour cell lines, but also primary tumour cells isolated from the ascites of patients with ovarian carcinomas were sensitive to the treatment with okadaic acid, an inhibitor of PP2A. Induction of apoptosis after okadaic acid treatment was accompanied by the phosphorylation of PKCζ, confirming a survival role of PP2A in ovarian cancer, and a potential pro-apoptotic function of PKCζ. Based on the in vitro cell culture work we analyzed how different members of the PKC family are regulated by H-REV107-1 or by the inhibition of PP2A activity with okadaic acid. Additionally, we verified an impact of the PI3-kinase pathway, a major survival kinase in ovarian carcinoma, in the regulation of PKC.

Analysis of novel PKCs revealed differences at the level of expression and phosphorylation. Thus, treatment with okadaic acid for 48 hours and overexpression of H-REV107-1 led to an increased expression of PKCε. Additionally, H-REV107-1 indirectly induced phosphorylation of the COOH-terminal residue Ser729, shown to enhance the enzymatic activity of PKCε (Parekh et al., 2000). This suggests that PKCε activity might be partially regulated in an H-REV107-1-dependent manner. Phosphorylation of Thr538 within the activation loop of PKCθ was elevated after 48 hours of treatment with okadaic acid and the AKT inhibitor LY294002, suggesting a negative but indirect regulation through PP2A and PI-3K. Additionally, Thr538 phosphorylation of PKCθ was increased in cells expressing H-REV107-1, suggesting a potential role of this kinase in H-REV107-1 signalling. The

phosphorylation of Thr505 located within the activation loop of PKCδ increased already 15 minutes after the addition of okadaic acid or LY294002, indicating that PKCδ is directly inactivated by PP2A and PI3K. Although the levels of total PKCδ seemed to be slightly increased after long-term okadaic acid and LY294002 treatment, the phosphorylation was strongly diminished. H-REV107-1 negatively regulated the expression of PKCδ, supporting the finding that PKCδ is not involved in H-REV107-1-dependent cell death. Expression of atypical PKCι was increased following 48 hours of treatment with okadaic acid, but neither phosphorylation nor total levels were affected by H-REV107-1.

To correlate phosphorylation of kinases in the activation site and their intracellular kinase activity, we applied in vitro kinase assay described in detail elsewhere (Nazarenko et al., 2010) and measured direct changes in the activity of PKCs upon okadaic acid treatment. A significant elevation of the PKCθ and PKCε activity was detected 24 hours after okadaic acid incubation, confirming that these PKCs, although not known to be direct PP2A targets, are negatively regulated by PP2A signalling in OVCAR-3 cells.

As inhibition of PP2A is required for H-REV107-1-dependent apoptosis, we next asked if these kinases might be involved in H-REV107-1-induced cell death and tested if the abrogation of PKCθ and PKCε activity impairs the proapoptotic function of H-REV107-1. OVCAR-3 cells were transfected either with the H-REV107-1 expression vector or with a control plasmid. Twelve hours later, the PKCθ- and PKCε-specific peptides were added. Caspase-3 cleavage was tested after 48 hours using Western blot analysis. H-REV107-1 expression resulted in the induction of caspase-3 cleavage, which was however not altered after peptide applications. Additionally, PKCθ-specific peptide treatment of control cells revealed a weak toxic effect. This result suggests that although PKCε and PKCθ are clearly activated in a PP2A and H-REV107-1-dependent manner, they are not essential for the H-REV107-1 proapoptotic activity in OVCAR-3 cells.

An important finding was that the atypical PKCζ is uncoupled from the PI3K pathway in ovarian cancer cells and is more likely to be a PP2A target. This is in contrast to the situation in the majority of normal and malignant tissues, in which PKCζ functions as an insulin-dependent PI3K effector. Importantly, overexpression of wild type H-REV107-1, but not of its PP2A interaction-deficient mutant, led to PKC phosphorylation, suggesting a direct link between the ability of H-REV107-1 to inhibit PP2A and the activation of PKCζ.

Electroporation of the ovarian carcinoma cells with PKCζ-expression plasmid demonstrated that high levels of this kinase are sufficient to induce apoptosis. In our work we demonstrated an increase of the sub-G1 cell population and caspase-3 cleavage. Molecular mechanisms by mean of which PKCζ induces apoptosis remained elusive and need further investigations. A recent work of Peng et al. might provide an additional hint for the mechanisms of PKCζ-dependent apoptosis (Chen et al., 2008). Using a mouse model, the authors demonstrated that PKCζ directly interacts with ERK1/2 in Kupffer cells, mediating a translocation of NF-kB into the nucleus and inducing its activity. The novelty of this finding is a direct link between PKCζ, EKR1/2 and NF-kB. Consistently, a cross-talk between NF-kB and PKCζ is well- characterised for many systems (Moscat et al., 2001;Moscat and az-Meco, 2011). Next, a potential interaction between PKCζ, ERK1/2, and NF-kB in ovarian cancer cells should be verified. A hypothetical scheme of PKC apoptotic cascade and cross-talk with other pathways is represented on the Fig. 5.
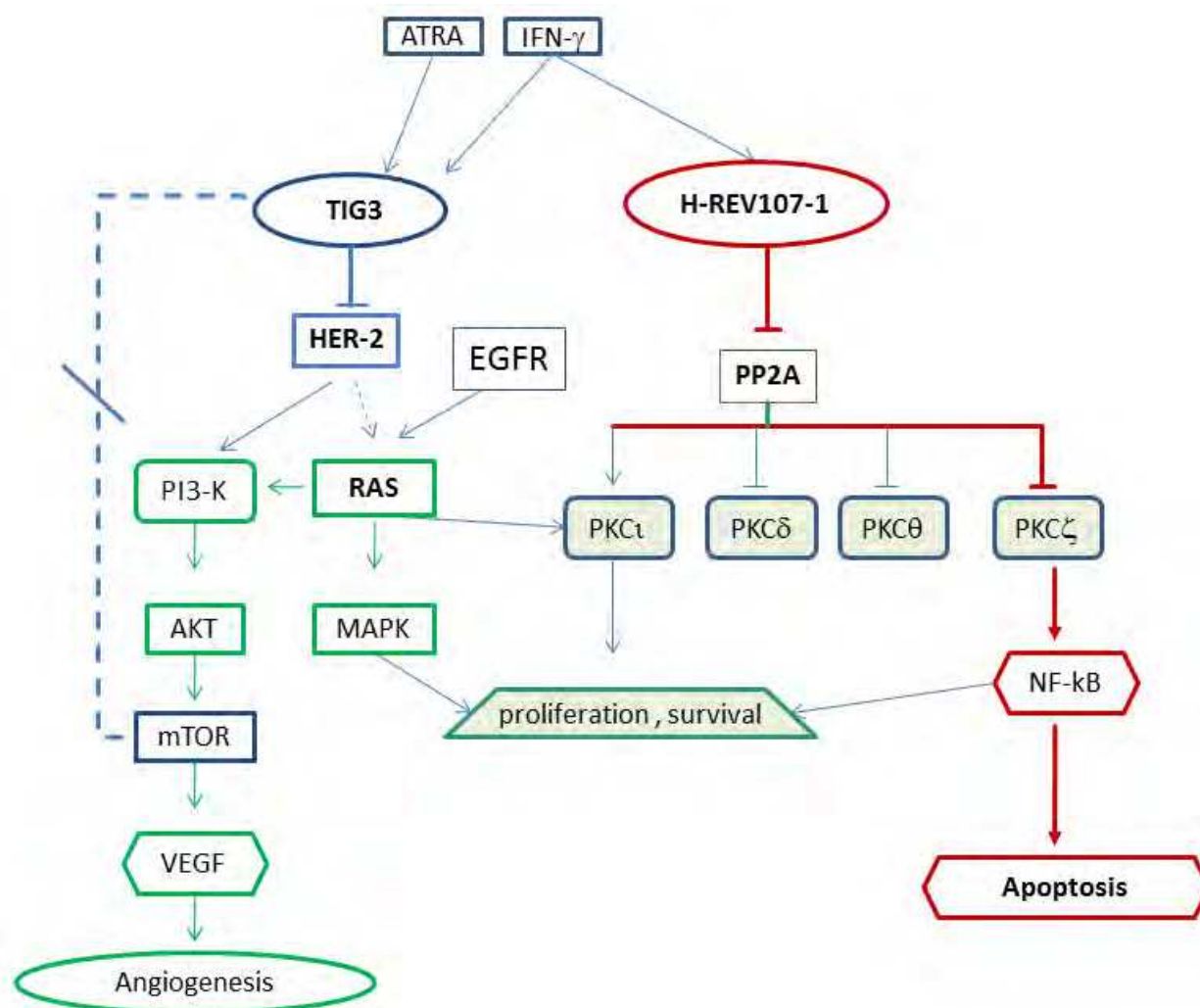
Fig. 5. Hypothetical scheme of the pro-apoptotic signal transduction network in ovarian cancer cells of two members of the HRS protein family, the H-REV107-1 (showed with red arrows) and TIG3 (showed with blue arrows). Both family members can be activated by IFNγ, whereas TIG3 can be additionally activated by ATRA. TIG3 mediates inhibition of HER-2, mediating herewith suppression of angiogenesis. H-REV107-1 inhibits PP2A, mediating activation of PKCζ. PKCζ functions as a major mediator of H-REV107-1-mediated cell death and is sufficient to induce apoptosis in a subset ovarian carcinoma cells, sensitive to the H-REV107-1--mediated apoptosis.

4. Receptor kinase pathway profiling in ovarian cancer cells

We applied the RPPA (reverse phase protein array) technique to define a potential regulation of PKCs by epidermal growth factor receptor inhibition. Earlier work on the H-REV107-1 tumour suppressor, an inhibitor of PP2A, has demonstrated that H-REV1071-1 is lost in a significant portion of ovarian tumours.

As a class II tumour suppressor gene, H-REV107-1 expression was reconstituted upon IFNγ treatment and MAPK inhibition and was able to induce specific phosphorylation of atypical PKC and the induction of apoptosis. These observations suggested to us that interference with oncogenic pathways might also have some impact on PKC isoform expression and/or

activation. Therefore, we asked whether PKCζ phosphorylation, which is necessary for apoptosis induction in ovarian cancer cells, might also be regulated by inhibitors and therapeutic agents that target mitogenic and survival pathways. The most prominent candidates for such an approach appeared to be the family of epidermal growth factor receptors (EGFR), whose members are frequently mutated and activated in human malignancies, and specific inhibitors are used for the treatment of ovarian carcinomas (De Marinis et al., 2002;Blank et al., 2005).

We performed a reverse phase protein array analysis (RPPA) of OVCAR-3 cells treated with the EGFR inhibitors Cetuximab and Gefitinib/Iressa, and tested the expression and phosphorylation of PKCζ and the expression of PKCα and PKCδ with antibodies established for this approach. To test if other signalling cascades are similarly affected following inhibition of EGFR signalling, we applied antibodies against phosphorylated AKT and ERK proteins and against the Bad protein.

This analysis showed an increased protein level of PKCζ and of its phosphorylated form 24 hours after the treatment with EGFR inhibitors. The RPPA analysis of ERK, AKT and Bad proteins revealed a moderate effect of EGFR inhibition on the phosphorylation status of these proteins and their expression. While total levels of Akt/PKB and phosphorylation of Bad Ser112 were unchanged, Akt/PKC and ERK phosphorylation were moderately increased after application of inhibitors. In addition to PKCζ, RPPA analysis also revealed elevated levels of PKCα and PKCδ following incubation with the EGFR inhibitors, suggesting a role of these kinases in EGFR downstream signalling.

Our experimental data obtained through profiling with reverse phase protein arrays revealed that application of Cetuximab or Gefitinib to OVCAR-3 cells induced only a moderate effect on MAPK and PI3K signalling, and had no effect onto cell growth. This suggests that specific targeting of EGFR is not sufficient to switch the survival program to an apoptotic program in these cells. In addition, EGFR inhibition led to a transient activation of PKCζ and to an up-regulation of PKCα and PKCδ. Neither PKCα nor PKCδ seem to play a crucial role in apoptosis induction in the cell lines tested, while we provided clear evidence for an involvement of PKCζ in the induction of apoptosis. The transient activation of PKCζ following EGFR interference was not sufficient to induce apoptosis. Therefore, the inhibition of oncogenic tyrosine kinase receptors might be a prerequisite for full or partial reconstitution of the players involved in apoptosis, but an additional trigger such as chemotherapy might be necessary to actually execute the death program (Nazarenko et al., 2010).

5. Conclusion

This chapter describes the impact of a family of tumour suppressor proteins, and the specific PKCζ-mediated signalling on apoptosis induction in ovarian cancer. The genes encoding H-REV107-1/HRSL3 and TIG3 both act as tumour suppressor genes. While the functional impact of TIG3 is still somewhat elusive, H-REV107-1 governs the decision between survival and apoptosis. Of major importance for the future research is the newly described function of H-REV107-1 and its related proteins, being phospholipases. This function indicates a specific role of lipid metabolism in the control of transformation and potentially tumour progression.

Furthermore, high expression levels of PKCζ and a correlation with poor prognosis were observed in human ovarian carcinoma samples and only the activation of endogenous PKCζ by okadaic acid or by the HRSL3 tumour suppressor, correlated with the induction of apoptosis in primary and immortalized ovarian carcinoma cells. This suggests a potentially inaccessible pro-apoptotic action of this kinase, which might be negatively regulated by activated tyrosine kinase receptors in ovarian cancer. In future research, identification of yet unknown substrates of the members of the HRS family will support current knowledge on the mechanisms of their pro-apoptotic function. Possibly, new aspects of functions, opening novel horizons in the therapy of ovarian cancer therapy, will be developed.

6. Acknowledgment

7. References

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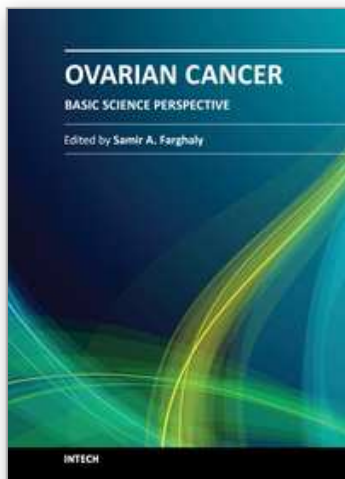
Ovarian Cancer - Basic Science Perspective

Edited by Dr. Samir Farghaly

Worldwide, Ovarian carcinoma continues to be responsible for more deaths than all other gynecologic malignancies combined. International leaders in the field address the critical biologic and basic science issues relevant to the disease. The book details the molecular biological aspects of ovarian cancer. It provides molecular biology techniques of understanding this cancer. The techniques are designed to determine tumor genetics, expression, and protein function, and to elucidate the genetic mechanisms by which gene and immunotherapies may be perfected. It provides an analysis of current research into aspects of malignant transformation, growth control, and metastasis. A comprehensive spectrum of topics is covered providing up to date information on scientific discoveries and management considerations.

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