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Involvement of the *ras* genes in female genital tract cancer (Review)

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Abstract. Human carcinogenesis is a multistep process involving complicated genetic events in which several oncogenes and oncosuppressor genes are implicated. The role of *ras* oncogenes in cellular transformation and apoptosis has been extensively examined and the dual role of *ras* as oncogene and oncosuppressor gene has been supported. Activation of *ras* occurs either by genomic alterations such as point mutations or by modulation of Ras protein expression. Many molecular and immunohistochemical studies have focused on *ras* activation in a wide range of human tumours. In this review, we summarize available information regarding the genomic and expression alterations of *ras* oncogenes in cervical, endometrial and ovarian cancer. Gynecological malignancies represent some of the most well-studied types of human cancer concerning *ras* activation and its possible use in clinical practice.

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1. Introduction

ras oncogenes and Ras protein. The family of *ras* oncogenes consists of three functional genes: H (Harvey)-*ras*, K (Kirsten)-*ras* and N (neuroblastoma)-*ras* which are located in chromosomes 11p15.5, 12p12.1 and 1p13, respectively (1). They have similar structure with a 5' non-coding exon and four coding exons (exons I-IV). The introns of the genes differ widely in size and sequence with the DNA sequence of 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 and is spliced into two isoforms: K-*ras*A and K-*ras*B (2).

The *ras* family oncogenes encode four highly related GTPases of 188 (K-RasB) or 189 (H-Ras, K-RasA, and N-Ras) amino acids in length. Different Ras proteins share high homology in the first 165 amino acids but show difference in 25 amino acids of the C-terminal region that constitutes the heterogeneous region. Their critical domains for GTPase function are present within the N-terninal 165 amino acids. All four proteins have a molecular weight of 21 kDa and are all termed ras p21 or Ras protein. Ras proteins are synthesized in the cytoplasm on free ribosomes as cytoplasmatic precursor proteins, undergo a series of post-translational modifications at the C-terminus including prenylation, proteolysis, carboxymethylation and palmitoylation and associate with the inner face of the plasma membrane (3,4). The membrane localization of Ras proteins is essential for their function.

ras signalling pathways. The Ras proteins cycle between an inactive GDP-bound state and active GTP-bound state at the plasma membrane (5). The ratio of GTP to GDP bound to Ras proteins is controlled by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), the enzymatic activity of which responds to extracellular stimuli such as growth factors (6). Binding of growth factors (EGF, PDGF, etc.) to their receptors at the cell surface leads to autophosphorylation of their tyrosine receptor kinases (7). Adaptor proteins such as growth factor receptor-bound protein 2 (GRB2) interact with receptor phosphorylated tyrosines. This allows recruitment of GEFs in the membrane where they promote the Ras transition into the Ras-GTP active complex. Ras is quickly inactivated by GAPs that stimulate GTP hydrolysis. Once active, Ras protein regulates different downstream signalling pathways by interacting with a plethora of target protein effectors (8).

The three main Ras effectors are Raf kinases, Ral-GEFs and PI3-K (Fig. 1). Raf family of protein serine/threonine kinases (A-Raf, B-Raf and Raf-1) initiate a kinase cascade that leads to ERK activation, which modulate both cytoplasmatic and nuclear transcriptional factors (9). Biological responses mediated by Raf/Ras activation include cellular proliferation,

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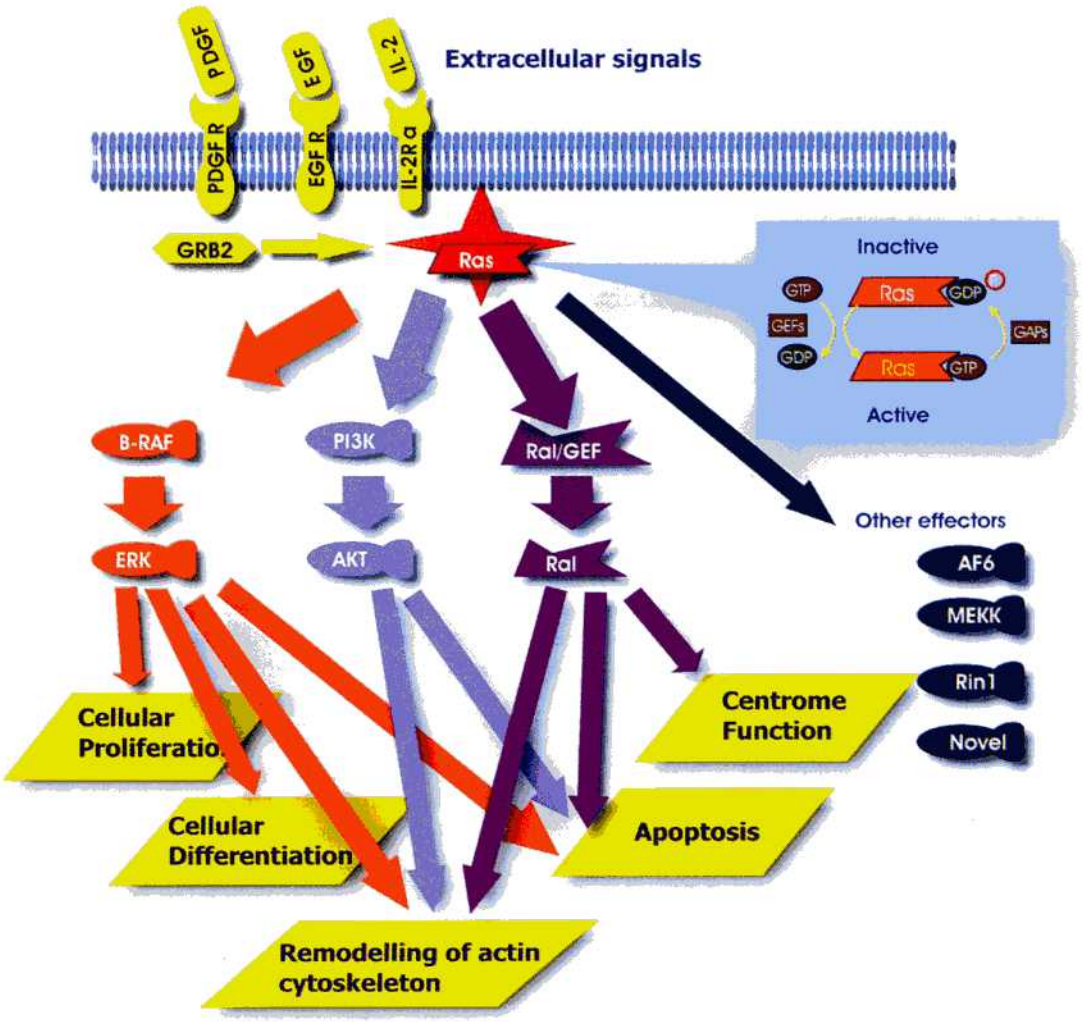

Figure 1. *ras* signalling pathways.

differentation, apoptosis (10,11), and cytoskeleton remodelling (12). Ral-GEFs activate the Ral GTPases RalA and RalB that contribute to the regulation of phospholipase D (PLD). Ras/Ral signalling affects specific gene transcription pathways and influence apoptosis, acting cytoskeleton and centrosome fuction (13-15). PI3-kinase initiates a kinase cascade leading to PKB/AKT activation that has been strongly connected with protection from apoptosis (16).

ras and human cancer. The family of *ras* oncogenes has been extensively studied for its involvement in the multistep process of carcinogenesis (17-19). Activation of *ras* oncogenes involves genomic alterations of *ras* oncogenes at the level of DNA as well as quantitative changes of the expression of Ras protein. *ras* activation is implicated in human carcinogenesis mainly by inhibiting apoptosis and promoting cellular proliferation. However, there is considerable evidence that activated *ras* oncogenes can also have opposite action by promoting apoptosis and inhibiting cellular proliferation and may have, under many circumstances, an oncosuppressive role. Early observations provided evidence indicating that expression of wild-type *ras* genes possesses anti-oncogenic properties. Proof that *ras* has oncosuppressive activity came very early when Spandidos and Wilkie demonstrated that expression of the normal H-*ras*1 gene suppresses the transformed phenotype induced by the T24 mutant H-*ras* (20) or the mutant N-*ras* (21) present in tumour cell lines. Recent development of the K-*ras*2 deficient animals provided the tool to study the role of wild-type K-*ras*2 gene in tumourigenesis (22). The dual role of *ras* as oncogenes and oncosuppressor genes has been recently reviewed (23).

The most common genomic alterations of the *ras* oncogenes are point mutations in codons 12, 13 and 61 which abolish p21 GTPase activity rendering p21 constitutively activated (24). Mutations of *ras* oncogenes are involved in a wide range of human tumours in various frequencies with tissue specificity depending on the member of the family which is activated. Mutations of K-*ras* occur more frequently in tumours of pancreas, lung and colon, H-*ras* in tumours of bladder and kidney while N-*ras* is mutated more often in melanoma and acute myelogenous leukaemia (25). Moreover, the presence of *ras* mutations has been associated with the clinical outcome of the patients and has been proposed as a negative prognostic factor in many types of cancer (26). Overexpression of *ras* oncogenes has also been detected in several human cancers including breast, colon, head and neck, bladder and lung and has been associated with the development of the disease (27). *In vitro* experiments have

shown that overproduction of even the normal Ras protein is sufficient to confer a transforming potential on cultured cells (28).

The gynaecological oncology field includes some of the most well studied types of human cancer as far as *ras* activation is concerned. It can be used as a positive and representative paradigm for the implication of *ras* oncogenes in human carcinogenesis. The purpose of this review is to summarize our knowledge concerning *ras* activation mediated either by genomic alteration events or by modulation of Ras protein expression in cervical, endometrial and ovarian tumours. We will also provide an overview of its relationship with clinicopathological parameters evaluating its possible usefulness in clinical practice.

2. *ras* and cervical cancer

Background. Cervical cancer is one of the most frequent malignancies in women worldwide (29). Clinical and epidemiological data have linked cervical cancer and cervical intraepethelial neoplasia (CIN) to the human papilloma virus (HPV) infection. Cervical carcinoma-associated HPV types 16 and 18 encode E6 and E7 oncoproteins which inactivate p53 and Rb altering cell cycle control and leading to chromosomal instability (30). However, the presence of HPV infection alone is not enough to cause tumourigenesis, suggesting a role for additional host-cell genetic factors such as activation of *ras* oncogenes.

Several transformation studies indicate that activation of *ras* oncogenes can transform cervical cells in cooperation with HPV (31,32). Di Paolo *et al* have demonstrated in nude mice that the addition of activated H-ras to HPV16-immortalized human cervical cells can result in malignancy (31). It has been also suggested that activated H-*ras* can overcome both the anti-proliferative and anti-transforming effects of p53, the most important HPV target (32). Recently, Gaiotti *et al* found that activation of *ras* promotes expression of HPV-16 E6/E7, induces cyclins A and B, and mediates growth stimulation of immortal keratinocytes by TNF-α (33). Furthermore, *ras* oncogenes appear to be involved in cellular response to radiotherapy indicating *ras* mutations as modulators of the effectiveness of radiotherapy in cervical cancer (34).

Genomic alterations of ras oncogenes

ras mutations. Point mutations of K-*ras* have commonly been implicated in cervical cancer with a frequency ranging from 0 to 61% (Table I). The vast majority of K-*ras* mutations occur in codon 12 including mostly transitions from GGT to GAT (35-41). It has been suggested that codon 12 K-*ras* mutations may serve as a molecular marker for the detection of the disease (37,38). However, other investigators have reported less frequent codon 12 K-*ras* mutations in cervical cancer indicating the need for larger epidemiological surveys (39-44). Mutations of H-*ras* at codon 12 have also been reported in cervical cancer, however, they occur less frequently than K-*ras* mutations in the same codon (37,38,45-47). For example, in the study of Dokianakis *et al*, which included 75 patients with squamous cervical carcinoma, codon 12 point mutations of the K-*ras* were detected in 24 (32%) patients while codon 12 H-*ras* mutations were detected in 6 (22%) patients (37). In the Wong *et al* study of 80 patients with squamous cervical carcinoma, codon 12 mutations of K-*ras* and H-*ras* were found in 49 (61%) and in 28 (35%) patients, respectively (38).

Table I. Mutational activation of *ras* family oncogenes in cervical cancer.

Author/refs.	Number of cases	*ras* gene	Frequency (%)
Sato *et al* (35)	7	K-*ras*	2/7 (28.6)
Falcinelli *et al* (42)	42	K-*ras*	0/42 (0)
Willis *et al* (49)	15	K-*ras*	1/15 (6.7)
Jiko *et al* (43)	25	K-*ras*	1/25 (4)
Koffa *et al* (36)	37	K-*ras*	10/37 (27)
		H-*ras*	3/37 (8.1)
		N-*ras*	2/37 (5.4)
Wong *et al* (38)	80	K-*ras*	49/80 (61)
		H-*ras*	28/80 (35)
Huang *et al* (45)	44	H-*ras*	8/44 (18.2)
Lee *et al* (46)	27	H-*ras*	6/27 (22)
Parker *et al* (39)	32	K-*ras*	3/32 (9.4)
Grendys *et al* (48)	33	K-*ras*	4/33 (12.1)
		H-*ras*	2/33 (6.1)
		N-*ras*	2/33 (6.1)
Ferguson *et al* (40)	27	K-*ras*	1/27 (3.7)
O'Leary *et al* (47)	20	K-*ras*	0/20 (0)
		H-*ras*	1/20 (5)
		N-*ras*	0/20 (0)
Aoyama *et al* (56)	20	H-*ras*	0/20 (0)
Dokianakis *et al* (37)	75	K-*ras*	24/75 (32)
		H-*ras*	7/75 (9.3)
		N-*ras*	0/75 (0)
Pochylski *et al* (44)	29	K-*ras*	0/29 (0)
Stenzel *et al* (41)	24	K-*ras*	2/24 (8.3)
Alonio *et al* (53)	39	H-*ras*	16/39 (41)

It is important to note that only a small number of studies have examined the frequency of *ras* mutations in other codons (38,39,41,42,44,46-49). Grendys *et al* identified *ras* mutations in 24.2% of specimens from 33 patients with early-stage cervical carcinoma. In this study, analysis at codons 12, 13, and 61 was performed, however, the detected mutations in H-, K-, and N-*ras* all occurred only at codon 61; 2 in H-*ras*, 2 in N-*ras* and 4 in K-*ras* (48). In the study of Parker *et al*, codon 61 K-*ras* mutations were detected in one out of 32 specimens with cervical cancer (39,42). Wong *et al* found K-*ras* codon 13 mutations in 5 out of 80 samples (6%) with invasive squamous cell carcinoma, while Willis *et al* found only one patient of 15 with cervical carcinoma to have a K-*ras* mutation and this was present in codon 13 (38,49). Polymorphisms in codon 31 have been detected in adeno-

carcinomas with high-risk HPV (16 or 18) from Korean women (50). Additional studies are needed to evaluate whether other codons such as codon 61, 13 and 31 represent characteristic 'hot-spots' of *ras* mutations in cervical cancer.

ras mutations and prognosis. Several studies have suggested association between *ras* mutations and the clinical parameters of the patients although conflicting results have been described (35,37-39,46). In the study of Dokianakis *et al*, K-*ras* point mutations were correlated with FIGO stage indicating that the presence of K-*ras* mutations coexists with the increase of the FIGO stage (37). Moreover, a negative correlation was found between the presence of K-*ras* mutations and the survival of the patients.

However, in other studies no correlation has been found between the presence of *ras* mutations and clinical stage or survival (35,37,38). Sato *et al* found no correlation between the presence of point mutation at codon 12 of K-*ras* and age, clinical stage, or depth of muscular invasion (35). In support, there were no significant differences in incidence of the H- and K-*ras* mutations among different histologic grades or clinical stages of cervical cancers studied by Wong *et al* (38). Cases with codon 12 H-*ras* mutations studied by Lee *et al* included 3 of 21 squamous, 2 of 3 adeno, and 1 of 2 adenosquamous carcinomas while no correlation was found between H-*ras* codon 12 mutations and patient survival time (46).

ras mutations and CIN. The presence of *ras* mutations in non-cancerous cytological samples indicates the possible role of *ras* mutations in premalignant cervical lesions. In patients with CIN, codon 12 mutations of K-*ras* were found in 4 out of 23 (17%), codon 12 H-*ras* mutations were found in 8.5%, while no sample carried a codon 12 point mutation of N-*ras* (51). Among 91 non-cancerous samples, 17.58% showed mutations in codon 12 of K-*ras* (52). In the study of Alonio *et al*, 20% of CIN III patients had patterns compatible with H-*ras* mutations (53). It has been suggested that mutational activation of K- and H-*ras* oncogenes is implicated in the development of CIN (51-54). Moreover, in H-*ras* mutated cases with CIN lesions, the progression took place in under two years, indicating that this detection may be an early predictive marker of rapid progression (53). It is important to note that *ras* mutations occur less frequently in CIN than in cervical cancer cases and that *ras* mutations have not been detected in cervical tissues with normal histology (47,55,56).

ras mutations and HPV. The simultaneous presence of *ras* activation by point mutations and HPV suggest a possible co-operation between *ras* activation and HPV infection in cervical cancer (40,41,52,57). The presence of HPV DNA in samples with cervical cancer has been correlated with the detection of codon 12 of K-*ras* mutations (40,52). Moreover, 'high-risk' HPV DNA coexists with K-*ras* oncogene alterations in a subset of moderately differentiated cervical carcinomas (41). Mouron *et al* demonstrated in cervical tissue samples with different grades of dysplasia a highly significant difference in K-*ras* codon 12 mutation frequency between 'high-risk' (HPV-16 and HPV-18) and 'low-risk' (HPV-6) HPV-infected samples (57).

Amplification or deletions of ras. Many studies have examined the presence of *ras* amplification in cervical cancer (45,58,59). In the analysis of Pinion *et al*, amplification of H-*ras* in CIN III and invasive cancer compared with normal cervix and CIN I was demonstrated, while in 20 cervical carcinomas studied by Huang *et al*, amplification rate of H-*ras* oncogene was 45% (45,58). However, no H-*ras* amplification was demonstrated in 38 patients with invasive cervical carcinoma studied by Iwasaka *et al* (59). Deletions of *ras* oncogenes have also been documented in cervical cancer. The c-H-*ras*-1 locus was analysed by Riou *et al* and shown to exhibit the loss of one allele in 36% of heterozygous cervical tumours (60). The H-*ras* allele deletions were present in 40% of codon 12 mutated cervical tumours.

Expression of ras oncogenes

Ras expression. It has been shown that the expression of Ras protein in cervical cancer is considerably enhanced compared with benign lesions or normal cervical tissues (58,61-66). The Ras protein levels detected immunohistochemically using the Y13 259 monoclonal antibody were elevated in malignant compared to benign human cervical lesions (63). Expression of Ras protein was noted in 51 of the 70 (72.8%) cervical carcinomas immunohistochemically using MAB-P21 as a probe, while there was no detectable Ras protein in the normal cervical tissues (64). Overexpression of Ras was found in 96% of 74 cervical carcinomas studied in relation to normal cervical epithelium from 10 patients with benign uterine leiomyomas (65). Expression of Ras protein in 32 cervical dysplasia/adenocarcinomas *in situ* lesions and invasive cervical adenocarcinomas was significantly higher than in 45 normal tissues (66).

In cell lines derived from Japanese patients with cervical carcinoma, Shirasawa *et al* detected H-*ras* mRNA expression at about nine times the level of that in normal cells (61). In the study of Shiratori *et al*, H-*ras* oncogene expression was often characterized by staining with a monoclonal antibody in cases with severe dysplasia, carcinoma *in situ* or invasive carcinoma while normal squamous epithelium was largely negative (62). In the analysis of 56 cervical tissues by Pinion *et al*, the expression of H-*ras* oncogene was elevated in invasive cancer compared with normal cervix, CIN I and CIN III (58). Recently, our group found that the transcript levels for H-*ras* and N-*ras* were significantly higher in cervical cancer cases compared to normal cervical tissues and CIN lesions indicating H- and N-*ras* up-regulation as a marker of malignant transformation in human cervical neoplasia (67). The exact role of differential mechanisms of H-, K- and N-*ras* oncogene transcriptional and translational activation in cervical tissue and their implication in Ras overexpression remain to be elucidated.

It has been reported that overexpression of Ras protein in squamous cervical carcinomas of different histologic types is detected more frequently in keratinizing and large cell non-keratinizing type than in small cell type (68). In the study of Huang *et al*, the expression of Ras protein in cervical carcinoma varied with grades of cell differentiation (64). The level of Ras expression in high-differentiation types was higher than that of the middle- and low-differentiation types.

Ras expression and prognosis. Overexpression of Ras protein has been related with various clinical parameters (46,65,66,69,70). Hayashi *et al* demonstrated that the patients with positive staining for Ras protein in cervical carcinomas have a higher incidence of lymph node metastasis than the patients with negative staining for Ras (69). In this study, although the levels of Ras protein expression in the metastatic sites were reduced compared to those in the primary sites, tumour cells in metastatic lymph nodes also expressed Ras. A significant relationship between Ras expression and risk of lymphatic spread was detected in early-stage cervical carcinoma proposing Ras positivity as an indicator of lymphatic spread (70).

However, Ras expression does not seem to be a significant predictor of prognosis. In the study of Lee *et al*, no correlation was found between H-*ras* oncogene expression and patient survival time (46). In FIGO stage I squamous cervical cancers studied by Garzetti *et al*, no connection was found between Ras expression and tumour size or histologic grade (70). Skomedal *et al* found no difference in aberrant expression of Ras protein related to histological type, grade of differentiation, FIGO stage, or relapse-free survival (65). No correlation was found by Leung *et al* between moderate/strong expression of Ras and stage at presentation or with survival (66). In the study of Sagae *et al*, although expression of Ras was related with prognosis of cervical cancers, the mode of the correlation was dependent on their histologic types (68).

Ras expression and CIN. Many studies have detected Ras protein in CIN lesions indicating its important role in early phase of carcinogenesis (71-75). Ras expression levels increase proportionally from CIN I to microinvasive cervical carcinoma and they have been suggested to be predictive of CIN progression (71,72). The frequency of positive Ras staining using anti-*ras* p21 mouse monoclonal antibody rp35 was 17.9% in CIN I, 28.9% in CIN II and 53.9% in CIN III, whereas in microinvasive carcinoma it was 50.0% (71). In the analysis of 204 cervical tissue samples including pre-malignant and malignant lesions as well as apparently normal cervical tissues, the immunoreactivity for N-Ras also increased with increasing histological abnormality from low grade squamous cervical lesions (SIL) to invasive carcinoma while none of the samples analysed displayed immuno-reactivity for H-Ras and K-Ras (72). However, in the immunohistochemical analysis of 395 biopsy specimens representing normal through CIN III histology, neither the proportion of tissues staining positive for Ras protein nor the staining pattern within the epithelial layers differed significantly among normal or CIN biopsy samples (75). Recently, our group found no statistically significant differences in mRNA transcript levels of H-, K-, and N-*ras* genes between 11 normal cervical tissues and 15 CIN lesions (67). Further research is required to evaluate Ras protein detection as a marker of CIN progression.

Ras expression and HPV. In the study of Pedroza-Saavedra *et al*, in serum samples from 38 healthy women and 55 women with different types of cervical lesions, patients positive for Ras antibodies were also positive for HPV E4 antibodies, suggesting an association between Ras expression and HPV (74). Giannoudis *et al* suggested that p53 status is correlated with Ras expression in low grade SILs infected with 'low, intermediate, and high-risk' HPVs (76). Moreover, it has been suggested that 'low, intermediate, and high risk' HPVs have different effects on both p53 and Ras protein expression. Understanding of molecular interaction mechanisms between Ras protein and HPV will enable us to evaluate the clinical usefulness of Ras protein detection in HPV-infected samples.

3. *ras* and endometrial cancer

Background. Endometrial cancer is the most common type of female cancer in the Western world (77). Endometrial carcinoma can be divided into two biologically and clinically distinctive subtypes of which one is estrogen-related (type I) and the other estrogen-unrelated (type II). Type I carcinomas occur at younger age, express estrogen (ER) and progesterone receptors (PR), are frequently associated with endometrial hyperplasia, show a good prognosis and histologically correspond to endometrioid carcinomas. Type II carcinomas occur at older age, are negative for ER and PR, arise in the background of atrophic endometrium, show poor prognosis and histologically correspond to serous carcinomas. A number of oncogenes and tumour suppressor genes are involved in the process of endometrial carcinogenesis (78).

The role of *ras* oncogenes in endometrial cell proliferation and differentiation has been examined by several *in vitro* studies (79-82). Expression of H-Ras has been associated with proliferation in transformed endometrial carcinoma cells and is increased by epidermal growth factor (EGF) and estradiol (79,80). Kato *et al* compared the growth response of endometrial carcinoma cells harbouring wild-type (Ishikawa cells) or mutated K-*ras* (HHUA cells) and demonstrated that the presence of mutated K-*ras* alone modulates the growth response of endometrial carcinoma cells to EGF (81). Moreover, *in vitro* experiments using a highly differentiated endometrial cancer cell line (SNG-II) have showed that tumour cell adhesion and infiltration decreased after exposure to conophylline, a new vinca alkaloid that inhibits *ras* oncogene expression (82).

Genomic alterations of ras oncogenes

ras mutations. Point mutations of K-*ras* in codon 12 have been reported to occur in 0-46% of endometrial cancers being more frequent than H-*ras* or N-*ras* mutations in the same codon (Table II). The most frequent codon 12 K-*ras* mutations are transitions from GGT to GAT, to GTT, to GCT, to AGT and TGT (35,83-85). K-*ras* point mutations in codons 13 and 61 occur less frequently (70,83,84,86-91). In Japan, Fujimoto *et al* found K-*ras* mutations in 10 (22.2%) out of 45 patients with endometrial carcinoma (92). Enomoto *et al* found in 19 endometrial adenocarcinomas, point mutations in codon 12 of K-*ras* were found in six tumours (32%), while only one mutation in codon 12 of N-*ras* and no mutation in H-*ras* (92). Caduff *et al* studied 112 patients from USA with carcinoma of the endometrium and K-*ras* codon 12 mutations were observed in 13 tumours (11.6%), including 11 endometrioid carcinomas, one

Table II. Mutational activation of *ras* family oncogenes in endometrial cancer.

Author/refs.	Number of cases	*ras* gene	Frequency (%)
Enomoto *et al* (83)	13	K-*ras*	6/13 (64)
Boyd *et al* (84)	11	K-*ras*	4/11 (36.4)
		H-*ras*	3/11 (27.3)
Sato *et al* (35)	21	K-*ras*	3/21 (14.3)
Enomoto *et al* (92)	19	K-*ras*	6/19 (32)
		H-*ras*	0/19 (0)
		N-*ras*	1/19 (5.3)
Mizuuchi *et al* (86)	49	K-*ras*	6/49 (12.2)
Ignar-Trowbridge *et al* (87)	30	K-*ras*	3/30 (10)
		H-*ras*	0/30 (0)
		N-*ras*	0/30 (0)
Fugimoto *et al* (85)	45	K-*ras*	12/45 (22.2)
Duggan *et al* (88)	60	K-*ras*	9/60 (15)
Caduff *et al* (93)	112	K-*ras*	13/112(11.6)
Enomoto *et al* (89)	38	K-*ras*	4/38 (11)
Jones *et al* (111)	32	K-*ras*	6/32 (18.8)
Schmitz *et al* (180)	21	K-*ras*	2/21 (10)
Varras *et al* (94)	55	K-*ras*	8/55 (15)
		H-*ras*	4/55 (7.3)
		N-*ras*	0/55 (0)
Semczuk *et al* (91)	13	K-*ras*	2/13 (15)
Esteller *et al* (96)	55	K-*ras*	8/55 (14.5)
Semczuk *et al* (97)	57	K-*ras*	8/57 (14)
Niederacher *et al* (98)	112	K-*ras*	13/112(11.6)
Semczuk *et al* (90)	82	K-*ras*	2/82 (2.4)
Lagerda *et al* (95)	58	K-*ras*	11/58 (18.9)
Mavani *et al* (99)	72	K-*ras*	3/72 (4.2)

undifferentiated carcinoma, and one carcinosarcoma (93). Additionally, in 38 endometrial adenocarcinomas from Colorado analysed by Enomoto *et al*, K-*ras* activation was detected in 4 cases (11%), three in codon 12 and one in codon 13 (89). Differences in the frequency of *ras* activation between populations from Japan and USA can be attributed to different epidemiological and geographical characteristics of these populations.

In Europe, studies conducted in Greek, Spanish, Polish and German populations have not shown significant differences concerning the frequency of *ras* mutations (94). In the study of Varras *et al* in the Greek population, K-*ras* mutations were detected in 8 out of 55 cases (15%) of primary endometrial carcinoma, H-*ras* in 4 (7.3%), while no mutations were found for the N-*ras* oncogene (94). In Spain, point mutations at codon 12 of K-*ras* were identified in 8 of 55 (14.5%) specimens with endometrial carcinoma while in another study K-*ras* mutations were detected in 11 (18.9%) of 58 endometrial carcinomas (95,96). In Polish women studied by Semczuk *et al*, mutations at exon 1 of the K-*ras* oncogene were detected in two of 13 human endometrial carcinomas (15%), while in another study, mutational activation in codon 12 of the K-*ras* gene was detected in 8 out of 57 (14%) endometrial carcinomas, whereas in codon 13 of the K-*ras* oncogene no point mutation was noted (91,97). In Germany, Niederacher *et al* studied 112 human endometrial carcinomas and K-*ras* mutations were detected in 11.6% (98). However, in 72 malignant samples of the human endometrium from Austria examined for point mutation in codons 12, 13 and 61 of the K-*ras* oncogene, a double mutation of codons 12 and 13 as a single-point mutation in one case of endometrioid carcinoma (2.8%) and two single-point mutations of codon 13 (5.6%) in two other cases of endometrioid carcinoma (2/72) were observed (99).

The frequency of K-*ras* mutations in endometrioid and serous endometrial carcinoma differ suggesting that different molecular genetic pathways are involved in the pathogenesis of these two common types of endometrial carcinoma (100-102). K-*ras* mutations at codon 12 were found in 15 of 58 (26%) endometrioid endometrial carcinomas and in only 1 of 45 (2%) serous endometrial carcinomas (100). Hori *et al* showed a tendency for cases with K-*ras* point mutations to be endometrioid rather than serous endometrial carcinoma (101). Among 58 endometrial carcinomas studied by Lagarda *et al*, all tumours with K-*ras* mutations were endometrioid carcinomas (95).

Mutations of K-*ras* have been associated with positive expression of progesterone receptor (PR) indicating that activation of K-*ras* may be involved in the development of hormonal independence in endometrial cancer (98). Zachos *et al* examined the activity of estrogen receptors (ER) and glucocorticoid receptors (GR) in human endometrial cancer and found increased binding of these receptors to H-*ras* element indicating the direct activation of H-*ras* by steroid hormone receptor binding (103). Transcriptional regulation of the H-*ras* oncogene by p53 protein in human endometrial tumours has also been demonstrated by Zachos and Spandidos (104). In this study overexpression of Ras protein was related to increased nuclear levels of wild-type p53 protein and elevated binding of the p53 protein to the H-*ras* element.

Esteller *et al* revealed a positive association of K-*ras* oncogene mutation and germline variants of the cytochrome P-450 1A1 (CYP1A1) gene, an estrogen-metabolizing gene associated with enhanced endometrial cancer risk (105). K-*ras* codon 12 point mutations as well as K-*ras* exon 2 point mutations have also been associated with expression of the retinoblastoma protein (pRB) (90,106).

K-*ras* mutations were more frequent in microsatellite instability (MI) positive than in MI-negative tumours supporting a close relationship between K-*ras* mutations and the phenomenon of MI in endometrial carcinomas (95). However, others have failed to find any relationship between MI and *ras* activation (107,108). Sakamoto *et al* found no relationship in endometrial carcinoma between MI and point mutations in K-*ras*, while in the study of Mutter *et al* K-*ras* mutations occured in both microsatellite stable and unstable premalignant endometrial neoplasia (107,108).

ras mutations and prognosis. Many studies have examined the relationship between the presence of K-*ras* mutations and various clinicopathological parameters suggesting an important role of activated K-*ras* in determining the aggressiveness of endometrial carcinoma (85,86,102). Mizuuchi *et al* found, K-*ras* activation to be an independent risk factor when compared with clinical stage, depth of myometrial invasion, and patient age (86). In endometrioid carcinoma cases, K-*ras* activation has been found to be responsible for more aggressive clinical behaviour in postmenopausal than in premenopausal patients (102).

In the study of Fujimoto *et al*, although no relationship appeared to be present between K-*ras* mutations and clinical stage, histological type, histological grade of differentiation, depth of myometrial invasion, and ascitic cytology, the positive rates of lymph node metastasis tended to be higher in the group with positive K-*ras* mutations than in the group without mutations (86). This is consistent with the results of Ito *et al*, who found that K-*ras* mutations were significantly associated with the presence of lymph node metastases (102). Alexander-Sefre *et al* have proposed molecular assessment of depth of myometrial invasion using K-*ras* mutation (109).

Other studies on human endometrial carcinoma have not found significant correlation of K-*ras* mutations with age, grade of differentiation, clinical stage, lymph node status and myometrial invasion (35,93,94,96-98,110). It has been proposed that the presence of K-*ras* mutations in endometrial carcinoma is not associated with patient prognosis and survival (93,94,111).

ras mutations and endometrial hyperplasia. K-*ras* mutations have also been observed in patients with endometrial hyperplasia, indicating that they may represent an early event in the development of endometrial cancer (88,92,112). K-*ras* mutations have been found in endometrial hyperplasias histologically classified as atypical and clinically considered premalignant. No detectable *ras* mutations in adenomatous hyperplasias and cystic hyperplasias have been found. Hachisuga *et al* reported the presence of K-*ras* mutations in codon 12 in tamoxifen (TAM)-related endometrial polyps (113). The incidence of mutations in codon 12 of K-*ras* in TAM-related endometrial polyps (64%) was greater than the incidence of the same mutations in sporadic endometrial hyperplasias, suggesting the important role of *ras* in chemically-induced transformation of endometrium in TAM-treated postmenopausal women with breast cancer.

Expression of ras oncogenes

Expression of Ras protein. Expression of Ras protein has been detected in endometrial carcinoma indicating that it may play an important role in the development of human endometrial carcinoma (63,114-117). It has been demonstrated that Ras protein is expressed in 68-75% of the specimens with endometrial carcinomas analyzed by immunohistochemical staining with monoclonal antibodies, while 47.5% has been found positive for H-Ras (115,116,118). The levels of Ras protein in human uterine lesions was studied as compared to normal tissue using an immunohistochemical assay (Y13 259 monoclonal antibody) and elevated expression of Ras in endometrial carcinoma was found (63). In this study, normal or atrophic endometrial mucosae were mostly negative while 6 out of the 12 hyperplastic endometrial lesions were found to be moderately or highly positive. Scambia *et al* studied 18 normal endometrial tissues and 37 human primary endometrial carcinomas by Western blot analysis and found that Ras levels were significantly higher in primary endometrial carcinomas than in normal proliferative tissues (114).

In contrast to other types of cancer (bladder, prostate, colon and breast) where Ras is detected only within neoplastic cells, in high-grade endometrial carcinomas Ras protein has also been detected within stromal cells (117). However, in the immunohistochemical analysis although most of the tumour cells expressed Ras protein, the stromal component was unreactive (114).

It has been found that the Ras protein levels are significantly higher in secretory than in proliferative endometrium (114). Estrogen receptor (ER)-positive tumours express higher Ras protein levels than ER-negative tumours, while a similar trend, although not statistically significant, has been found between Ras values and progesterone receptor (PR) expression (114). In immunohistochemical analysis of Ras protein using monoclonal antibody rp-28 by Yaginuma *et al*, Ras expression was related to the histological type of endometrial cancer. In this study, 63% of well differentiated adenocarcinomas, 53% of moderately differentiated and 40% of poorly differentiated were positive, while the staining intensity of Ras seemed to be stronger in the more differentiated types of endometrial carcinoma (119). Among patients with endometrial cancer, expression of Ras protein has been detected more frequently in post-menopausal than in premenopausal women indicating the existence of different carcinogenetic mechanisms in these two groups of patients (119).

Ras expression and prognosis. Expression of *ras* oncogenes has been examined in relation to patients prognostic parameters with conflicting results (115,116,118,120). In the study of Miturski *et al*, the simultaneous detection of both Ras and p53 proteins was correlated with advanced clinical stage of human endometrial carcinoma (118). Semczuk *et al* showed that Ras immunostaining was related to myometrial invasion (116). However, no significant relationship between Ras expression and patients survival has been proposed in other studies (115,120).

4. *ras* and ovarian cancer

Background. Ovarian cancer is the leading cause of death from gynaecological malignancies and the fifth most common cause of cancer death among women (121). There is evidence that ovarian cancer may be derived from the progressive transformation of benign and/or borderline tumours (122). Benign ovarian tumours lack the cytological and nuclear atypia and follow a benign clinical course. Borderline ovarian tumours retain a cellular and nuclear architecture similar to invasive carcinomas, without histological evidence of stromal invasion, but with the ability to metastasize. Mutations involving different oncogenes and tumour suppressor genes accumulate during the process of malignant transformation of ovarian cells.

The role of *ras* oncogene activation in ovarian carcinogenesis has been extensively studied (123-128). *In vitro* experiments have demonstrated that transfection of mutant H-*ras* into immortalized ovarian cells can induce malignant transformation (123). Yang *et al* found that silencing of H-*ras* oncogene expression using small inhibitory RNA (siRNA) in ovarian cancer cell lines decreases transformation efficiency and tumour growth (124). Moreover, it has been shown that activation of AKT2, a member of protein kinase B family activated via *ras* signalling pathways, is a common occurrence in human ovarian cancer (125,126). Thangaraju *et al* found that BRCA1 overexpression in ovarian cancer cell lines enhanced signalling through a pathway that involved H-*ras* activation (127). It has been demonstrated that hepatocyte growth factor (HGF), a multifunctional growth factor which has several biological effects on epithelial cells, such as proliferation, invasiveness and morphogenesis modulates motility and invasiveness of ovarian carcinomas via *ras*-mediated pathways (128).

Genomic alterations of ras oncogenes

ras mutations. Mutations of the K-*ras* oncogene in ovarian cancer range from 4 to 47% while H-*ras* and N-*ras* oncogenes occur less frequently (Table III). The most frequent codon 12 K-*ras* mutations are GGT to GAT transversions (129). For example, in the study of Park *et al* in 37 ovarian cancers, the incidence of codon 12 mutations of K-*ras* gene was 35.1% (13/37) and the distributions of transversions from GGT to GAT, to AGT, to TGT, and to GTT were 32.4% (12/37), 2.7% (1/37), 0% and 0%, respectively (130).

Mutations of *ras* oncogenes in other codons such as 13 and 61 have also been implicated less frequenlty in ovarian cancer (131-133). In 28 tissue specimens of human ovarian cancer examined by Chien and Chow, one specimen was found with a H-*ras* point mutation at codon 12, two had a K-*ras* mutation at codon 12, one had a K-*ras* mutation at codon 13 and none at codon 61 (132). Hogdall *et al* analyzed the presence of mutations at codons 12 and 13 of the K-*ras* oncogene in 138 women with invasive ovarian cancer and K-*ras* codon 12 mutations were found in 8.7% of the patients while a K-*ras* codon 13 mutation was found in 1.5% (133).

Peritoneal fluid evaluation forms part of the staging process of ovarian cancer. However, since cytological examination may be negative in the presence of micrometastatic ovarian cancer, molecular analysis of peritoneal fluid can be used to complement current conventional diagnostic procedures for detection of primary ovarian cancer (134). Genetic abnormalities, such as genomic alterations of K-*ras* oncogene present in cancer cells can be detected using molecular biology techniques and can increase the diagnostic accuracy of peritoneal fluid evaluation (135). In 1999 it was suggested that K-*ras* detection in peritoneal fluid may have value for the early diagnosis and monitoring of ovarian cancer (136). This study demonstrated a high incidence of K-*ras* gene mutations in the peritoneal washings or ascites of women with ovarian adenocarcinomas related to FIGO staging whereas K-*ras* mutations in cystadenomas peritoneal fluid were less frequent. However, Parrella *et al* studied 14 ovarian cancers and matched peritoneal fluid but no K-*ras* mutation was detected either in tissues or in peritoneal fluid (137). Further studies are required to determine the sensitivity of the detection of K-*ras* mutations in peritoneal fluid and their importance for the early diagnosis of ovarian cancer.

Table III. Mutational activation of *ras* family oncogenes in ovarian cancer.

Author/refs.	Number of cases	*ras* gene	Frequency (%)
Enomoto *et al* (83)	13	K-*ras*	2/13 (15.4)
Enomoto *et al* (131)	37	K-*ras*	10/37 (27)
		H-*ras*	0/37 (0)
		N-*ras*	0/37 (0)
Chien *et al* (132)	28	K-*ras*	3/28 (10.7)
		H-*ras*	1/28 (3.6)
Teneriello *et al* (129)	25	K-*ras*	1/25 (4)
Ichikawa *et al* (138)	32	K-*ras*	8/32 (25)
Park *et al* (130)	37	K-*ras*	13/37 (35.1)
Tanimoto *et al* (148)	17	K-*ras*	4/17 (23.5)
Cuatrecasas *et al* (144)	97	K-*ras*	34/97 (35.1)
Mandai *et al* (149)	10	K-*ras*	4/10 (40)
Haas *et al* (150)	17	K-*ras*	2/17 (11.8)
Caduff *et al* (151)	81	K-*ras*	9/81 (11.1)
Varras *et al* (142)	48	K-*ras*	11/48 (22.9)
		H-*ras*	3/48 (6.3)
		N-*ras*	0/48 (0)
Dokianakis *et al* (37)	47	K-*ras*	22/47 (46.7)
		H-*ras*	3/47 (6.4)
		N-*ras*	1/47 (2.1)
Suzuki *et al* (139)	64	K-*ras*	15/64 (23.4)
Schmitz *et al* (180)	21	K-*ras*	2/21 (9.5)
Hongdall *et al* (133)	138	K-*ras*	14/138 (10.1)
Okuda *et al* (141)	64	K-*ras*	7/64 (10.9)
Singer *et al* (140)	31	K-*ras*	0/31 (0)

The incidence of K-*ras* mutations is significantly higher in cases of mucinous than serous ovarian carcinoma (83,131,133,138-140). K-*ras* mutations have also been detected more frequently in patients with ovarian clear cell adenocarcinoma than in patients with ovarian endometrioid adenocarcinoma (141). The incidence of codon 12 K-*ras* point mutations was higher in patients with serous cystadenocarcinomas than in patients with mucinous cystadenocarcinomas (130). However, in another study K-*ras* mutations were not associated with the differentiation of the epithelial cells (142). The exact involvement of K-*ras* mutations in ovarian differentiation remains to be elucidated.

ras mutations and prognosis. Many studies have examined the presence of K-*ras* mutations in relation to clinicopathological parameters in order to evaluate the prognostic role of detection

of *ras* mutations in ovarian cancer. Point mutations of K-*ras* were found most frequently in patients with advanced stage disease and in those with pelvic lymph node metastases indicating K-*ras* mutations as a marker that can predict a high risk of pelvic lymph node metastases in ovarian cancer (130). The presence of K-*ras* gene mutations was statistically correlated with FIGO and surgical stage of the malignant specimens (136). It has been proposed that ovarian cancers of mucinous and non-mucinous histology are significantly different with respect to clinical characteristics, survival and molecular alterations (143). However, most of the researchers have demonstrated no correlation between *ras* mutations and patients survival (133,141,142,144). No significant correlation was found between *ras* mutations and clinicopathological parameters or clinical outcome of primary invasive ovarian carcinomas in Greek population (142). The presence of K-*ras* point mutations did not correlate with survival in non-mucinous ovarian epithelial tumours (144). K-*ras* mutations did not affect survival of patients with ovarian clear cell or ovarian endometrioid adenocarcinoma (141), and the frequency of K-*ras* mutations was correlated with the histological type of tumour, but not with age, stage, radicality of operation and patients survival (133).

ras mutations and benign/borderline ovarian tumours. Ovarian cancers are likely to arise through malignant transformation of benign ovarian tumours (122). The results of many studies confirm that K-*ras* mutations occur in benign and borderline ovarian tumours supporting that K-*ras* mutational activation is an early event in ovarian carcinogenesis (129,133,138,144-155).

It has been suggested that the frequency of K-*ras* mutations increases from benign to borderline tumours and from borderline tumours to ovarian cancer (122,138,144,147). In the study of Ichikawa *et al*, mutations of K-*ras* in codon 12 were detected in 4 of 30 mucinous adenomas (13%), in 4 of 12 mucinous borberline tumours (33%), while the incidence of K-*ras* mutations in mucinous carcinomas was 46% (138). Cuatrecasas *et al* found codon 12 point mutations of K-*ras* in 55.7% of mucinous cystadenomas, 73% of borderline tumours and 85% of ovarian carcinomas, while codon 13 mutations were detected in five cystadenomas, three borderline tumours and three carcinomas (147). In a series of 144 non-mucinous ovarian tumours, the frequency of K-*ras* mutations at codons 12 or 13 was 20% (7/35) in benign tumours, 25% (3/12) in borderline tumours, and 35% (34/97) and in carcinomas (144).

Higher incidence of K-*ras* mutations in borderline tumours than ovarian cancer have been reported suggesting the essential role of K-*ras* mutations in borderline tumours (133,145,148,150,151).

Mutations of K-*ras* have been detected at a higher frequency in mucinous borderline tumours compared to serous borderline tumours (145,146,149,151,153). It is suggested that K-*ras* mutations occur during the transformation from benign cystadenomas to mucinous borderline tumours or mucinous carcinomas, providing molecular genetic support for the hypothesis of the 'adenoma-carcinoma sequence' in mucinous ovarian tumours. Mucinous borderline tumours represent precursors of mucinous carcinoma of the ovary, while serous borderline tumours, although they have some molecular features associated with malignancy, are unlikely to represent a precursor of invasive serous carcinoma (122).

Amplification and deletions of ras oncogenes. Amplification of the *ras* oncogenes has been widely studied in ovarian cancer (156-162). Amplification of H-*ras* and K-*ras* has been detected in ovarian carcinomas in a small number of cases but compared to tumours in other anatomical sites it has been proposed to be specific to ovarian tumours (157). Amplification of K-*ras* was found in 3 of 7 cases and amplification of H-*ras* in 1 of 12 cases with ovarian adenocarcinoma (158). The amplification rate of N-*ras* and K-*ras* in ovarian carcinomas was 44 and 31%, respectively (159), and took place chiefly in cases of early stage and those of good differentiation. Corresponding elevated levels of c-K-*ras2* mRNA and Ras protein were found in tumours with K-*ras2* amplification (156). The levels of K-*ras* specific RNA transcripts and Ras protein were also related to elevated amplification of K-*ras* (163). However, it has been suggested that amplification of the K-*ras* oncogene is not associated with tumour progression and metastasis (160).

Allelic loss of *ras* oncogenes or chromosomes where *ras* oncogenes are located have also been reported in ovarian cancer and may be involved in ovarian cancinogenesis. Allelic deletions of the H-*ras* oncogene have been found to be a very common abnormality in human ovarian adenocarcinomas and have been used as markers of larger chromosomal deletions on chromosome 11 (164). Chromosome 11 is one of the most commonly involved chromosome in ovarian cancer, reported abnormal in 83% of 29 tumours (165). Allele loss on chromosome 11 is present in late stage human ovarian carcinomas and has been associated with poor patient survival (166,167).

Expression of ras oncogenes

Ras protein expression. Overexpression of *ras* oncogenes only occurs in a small proportion of ovarian carcinomas but may have an important role in the progression of the disease pattern (146,148,168-174). In 57 tumour specimens from patients with advanced ovarian cancer, although the pattern of the staining was similar to the germinal epithelium of normal ovaries, the intensity of staining was more enhanced in carcinomas than in normal ovaries (168). Overexpression of *ras* oncogenes was found in 16 of 80 (20%) patients with epithelial ovarian cancer (170). Levels of Ras protein detected were similar in normal and cystic ovaries and in benign tumours, whereas they were significantly higher in malignant tumours than in control tissues (172). Compared to normal ovaries and benign ovarian tumours, higher levels of Ras protein were also found in 45% of the 100 ovarian carcinomas (173).

The presence of Ras overexpression in borderline ovarian tumours suggests that it may be an early genetic alteration in ovarian tumourigenesis (148,169,174-176). Ras expression was relatively higher in serous borderline tumours and papillary serous cystadenocarcinomas in contrast to normal ovary and serous cystadenomas (176). It has been suggested that Ras expression could be an indicator of malignant potential, enabling us to distinguish benign lesions from borderline ovarian tumours and carcinomas.

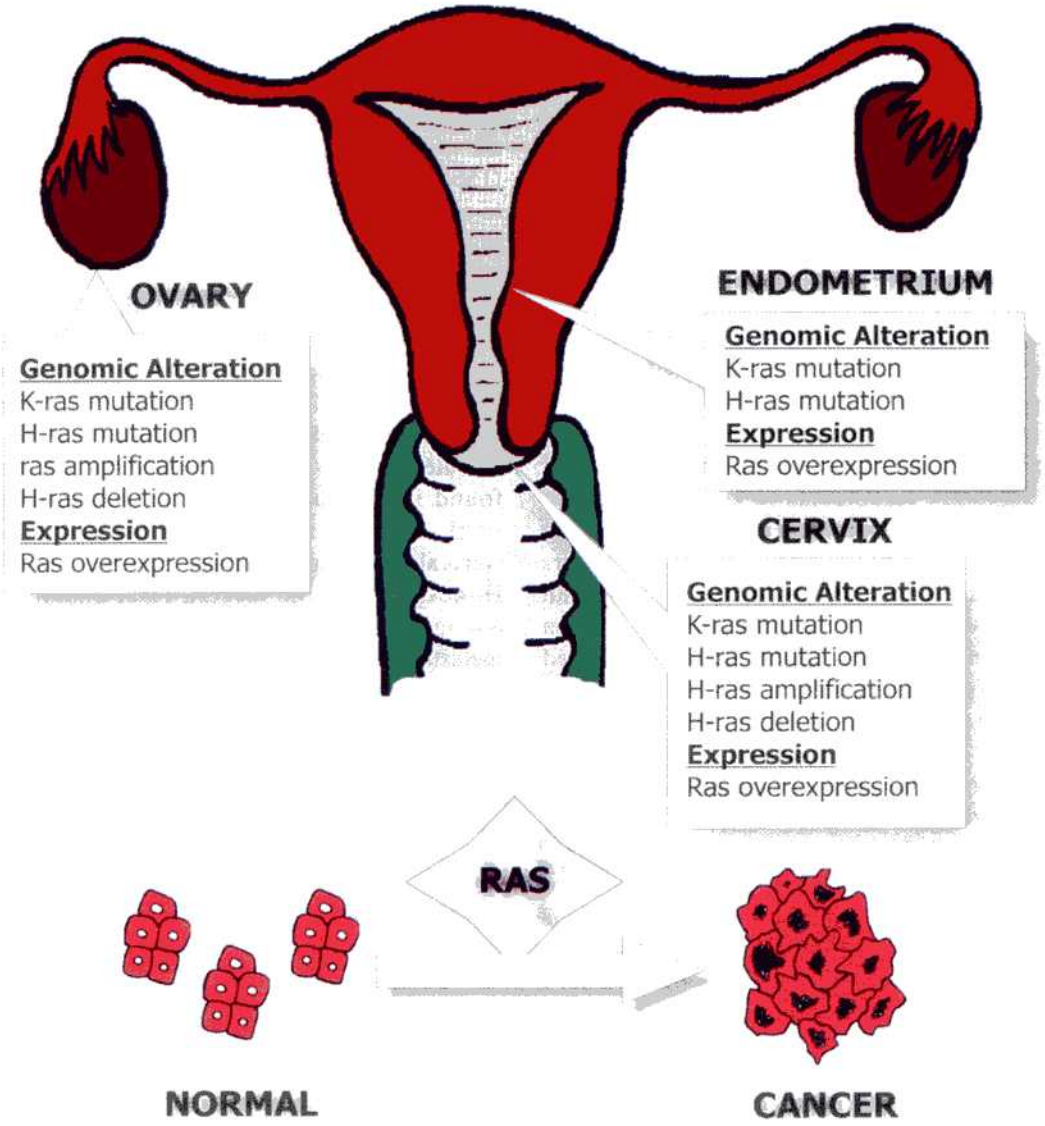

Figure 2. Involvement of the *ras* family oncogenes in female genital tract carcinogenesis.

Overexpression of Ras protein has been observed in serous and mucinous cystadenocarcinomas, undifferentiated adenocarcinomas, and clear cell carcinomas but not in germ cell and sex-cord stromal tumours (171). This differential expression of *ras* p21 has been suggested to be due to the different histogenesis of ovarian tumours reflecting a different carcinogenic mechanism for different types of malignancy.

Statistical analyses have shown a strong positive correlation between cyclin D1 and K-Ras immunoexpression in ovarian cancer cell lines and tumour tissues (177). It has also been found that estrogen receptor (ER)-positive and progesterone receptor (PR)-positive tumours express higher Ras protein levels than ER-negative and PR-negative tumours (172). Suto *et al* showed that tumourigenic transformation induced by the *ras* oncogenes is associated with alterations in estradiol biotransformation (178). Zachos *et al* found increased binding of glucocorticoid receptor (GR) to H-*ras* GR element and of estrogen receptor (ER) to H-*ras* ER element in all ovarian tumours tested (179). It has been suggested that steroid hormone receptor binding could directly activate the H-*ras* oncogenic potency in ovarian lesions. Overexpression of the Ras protein in ovarian tumours has also been correlated with elevated binding and increased nuclear levels of wild-type p53 (104).

Ras expression and prognosis. The levels of Ras expression have been found significantly higher in tumours of patients with recurrent or persistent disease after chemotherapy compared to tumours of patients at initial presentation (170). Moreover, Ras protein levels are higher in metastases than in primary ovarian carcinomas (146). A significant relationship has been shown between Ras positivity and a shorter overall survival and progression-free survival (146,172). Simultaneous overexpression of p185 and Ras protein has also been associated with shorter disease-free and overall survival (173).

No association between Ras protein and the histologic characteristics, the clinical stage and the patients prognosis have also been shown (168,169,174). There was no correlation between staining intensity of Ras protein and the histologic type, the histologic grade, the ploidy class, and the clinical outcome (168). Ras expression was comparable in

I/II and III/IV FIGO stages (169) and no difference in Ras expression was observed between the cases examined in spite of the differences in the degree of differentiation of the epithelial ovarian carcinomas (174).

5. Future perspectives

A major concern regarding gynaecological cancer is the lack of specific tumour markers for early detection, for accurate prediction of biological behaviour and for accurate assessment of prognosis. From this point of view *ras* oncogenes allow us to improve our understanding of disease aetiology (Fig. 2), and provide more precise diagnostic and prognostic characterization of individual tumours. The evaluation of *ras* activation in patients with cervical intraepithelial neoplasia, endometrial hyperplasia and benign or borderline ovarian tumours may be used to identify those at high risk for developing carcinomas. Differences in the incidence of K-*ras* gene mutations in various ethnic groups or geographic regions may indicate the presence of genetic or environmental factors influencing the pathway of female genital carcinogenesis. The involvement of *ras* oncogenes in radioresistance or multidrug resistance will result in the improvement of potential therapies that can be targeted at *ras* oncogenes, through approaches such as selective inhibition by anti-sense oligonucleotides. The potential *ras* oncogenes to be used as therapeutic targets in gynaecological cancer will require further research.

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