Immunohistochemical tumour markers in endometrial carcinoma

E. Ioachin, *M.D.*

Pathology Department, University of Ioannina, Medical School, Ioannina (Greece)

Summary

Endometrial adenocarcinoma is the most common malignant neoplasm of the female genital tract and, despite its relative frequency, the molecular events that contribute to the development and progression of the lesion remain poorly understood. The normal human endometrium is characterizel by hormone-dependent variations during the menstrual cycle. This tightly controlled system is disturbed in endometrial hyperplasia and carcinomas and a series of changes initiate and promote progression towards the malignant phenotype. These changes can be subdivided into discrete steps, involving activation of oncogenes, inactivation of tumour suppressor genes, deregulation of cell cycle regulators or other proteins involved in tumour invasion and progression. Immunohistochemical expression of different biomarkers such as hormone receptor status (ER, PR), proliferation associated indices (PCNA, MIB1), oncogene (c-erbB-2), tumour suppressor gene products (pRb, p53 protein), cell cycle related proteins (cyclin D1, cyclin E, p21/WAF1), anti-apoptotic protein (bcl-2), adhesion molecule (CD44s), proteolytic enzyme (cathepsin D), heat shock protein (hsp27) and metallothionein (MT) has shown the contribution of these molecules to endometrial carcinogenesis in a hormone-dependent or independent manner as an early or late event. In addition, these biomarkers seem to be correlated with tumour differentiation or myometrial invasion, and therefore could be considered as indicators of the biological behaviour of endomertial carcinoma. Furthermore, the interrelationships of these molecular markers show that these genetic dysregulations could be implicated in the control of cell proliferation and differentiation, and thereby in the multistep process of endometrial carcinogenesis.

Key words: Endometrial carcinoma; Immunohistochemical markers; Hormone receptoprs; PCNA, MIB1; c-erbB-2; pRb; p53; Cyclin D1; Cyclin E; bcl-2; CD44s; Cathepsin D; hsp27; Metallothionein.

Introduction

Endometrial carcinoma represents the most common invasive gynaecological malignancy in many parts of the world. Based on clinicopathological observations Bokhman [1] proposed that there are two main types of endometrial carcinoma: type 1 tumours related to hormonal imbalances and type 2 tumours that seem largely unrelated to estrogen. According to this model, type 1 tumours are indolent neoplasms that are associated with hyperlipidemia, obesity, and signs of hyperestrogenism, such as anovulatory bleeding, infertility, late menopause and endometrial and ovarian stromal hyperplasia. Type 2 tumours are unrelated to these features, behave aggressively and lack the progesterone responsiveness of type 1 tumours. It has been suggested that the majority of type 1 tumors correspond to the endometrioid type of endometrial carcinoma, whereas type 2 tumours probably include most serous carcinomas and some other aggressive types [2]. It has been proposed that serous carcinomas develop from "endometrial intraepithelial carcinoma", whereas endometrioid carcinoma and endometrial hyperplasia are associated with microsatellite instability and *ras* and *PTEN* mutations, and abnormal accumulation of p53 protein [3]. A number of pathological features have been identified as predictors of the clinical course of endometrial cancer. Surgical stage, histological grade and subtype, myometrial penetration, nodal involvement and peritoneal cytology have served as relatively reliable prognostic factors.

Biological markers have become increasingly important since additional variables are needed to give information that can lead to a better understanding of the biology of the carcinoma and give a biological basis for treatment. Various techniques are involved including biochemical and immunohistological markers and, more recently, cytogenetic and molecular biological techniques. The main advantage of the immunohistochemical technique is the application to routinely processed material in correlation with transitional morphological parameters. Despite the extensive research that has alteady been performed in this area, the results are conflicting. These discrepancies may possibly be attributed not only to the genetically different incidences of the biological markers, which have been studied in various populations, but also to differences in the evaluation, standardization and interpretation of the results. The immunohistochemical markers studied can be divided into the following groups: 1) hormone receptors (ER, PgR) 2) markers related to proliferative activity (Ki-67, PCNA) 3) oncogenes and tumour suppressor genes products (c-erbB-2, p53, rRb), 4) cell cycle regulatory proteins (cyclin D1, cyclin E, p21/WAF1), 5) apoptosis-related protein (bcl-2), 6) adhesion molecule marker (CD44s), 7) proteolytic enzyme (cathepsin D) and 8) other markers (hsp-27, metallothionein).

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Hormone receptors (ER, PR)

Endometrial tissue is considered to be a sex steroid-dependent tissue and immunohistochemical analysis studies have reported expression of hormone receptors in normal, hyperplastic and neoplastic endometrium [4-7]. Although the importance of ER and PgR has not been as well established as in breast carcinomas, it has been suggested that the ER/PgR status in gynaecological tumours, especially in endometrial carcinomas, may have prognostic significance [5]. The ER/PgR status, especially PgR, has been well correlated with histological differentiation and patient survival. Indeed positive ER/PgR has been detected more frequently in well-differentiated carcinomas [8] and is associated with a better prognosis [9]. In our previous studies estrogen and progesterone receptors were detected in 22.2% and 20.99% of carcinomas, respectively. This expression was lower in carcinomas in comparison to hyperplasias and the normal proliferative endometrium. The hormone receptor status was correlated with the proliferative associated index (Ki-67), with pRb and hsp27 [10, 11].

Markers related to proliferative activity (Ki-67, PCNA)

Cell kinetic data are an important adjunct to histologically-based tumour classifications and provide reliable information about future tumour behaviour. The endometrium is an actively proliferating tissue and there is an overlap in the cell proliferation fraction between benign endometrium and endometrial carcinoma. Previous studies of proliferation markers in endometrial carcinomas have obtained conflicting results regarding their usefulness with regard to prognosis. Determination of the Ki-67 index in a series of normal, hyperplastic (with atypia and without atypia) and malignant endometrial lesions showed no evidence of correlation with progression to malignancy [12]. A high PCNA index has been associated with advanced cancer stage, myometrial invasion and c-erbB-2 expression [13]. In a previous study, the Ki-67 and PCNA score was higher in carcinomas in comparison to the cases of hyperplasias and normal proliferative endometrium and increased expression of proliferation markers was correlated with tumour grade. In addition, the expression of proliferation indices was correlated with pRb [10].

Oncogenes and tumour suppressor genes (c-erbB-2, p53, pRb)

Several genetic aberrations have been implicated in tumorigenesis including activation of cellular protooncogenes or inactivation of tumour suppressor genes.

C-erbB-2 oncoprotein is a member of the type I family of receptor tyrosine kinases which is involved in the regulation of growth of many kinds of normal tissue [14].

Overexpression of c-erbB-2 protein is a frequent and prognostically relevant event in a variety of human cancers. In tumours of hormonally sensitive tissues, such as endometrial carcinoma, c-erbB-2 protein expression has been correlated with advanced stage disease [15] even though no correlation with histological grade or stage has been found in other reports [14]. In a previous study [10], overexpression of c-erbB-2 (> 25% positive tumour cells) was detected in 11.8% of the carcinomas, while the cases of hyperplasias and the normal endometrium showed absence or very low expression. This overexpression was correlated with tumour grade and the proliferative activity as estimated with both proliferative indices, PCNA and Ki-67.

p53

Inactivation of the *p53* function is well accepted to be a key event in tumorigenesis.

A growing list of p53-regulated target genes has been identified, through which p53 is involved in pathways of cell cycle control, angiogenesis, DNA repair, differentiation, growth factor signaling and apoptosis. Mutations in p53 can increase its protein stability, resulting in a prolonged half-life and detectable nuclear accumulation by immunohistochemical methods. Alterations to the human *p53* gene, either in the form of gene loss or mutations, have been observed to be a common change in endometrial carcinomas related to tumour development and progression [16-21]. Immunohistochemical overexpression of p53 has also been reported to be related to high-grade and advanced tumour stage [13, 22-24] as well as with decreased patient survival [22, 25, 26]. In our previous study [10], nuclear expression of p53 protein (> 5% positive tumour cells) was detected in 17.2% of the carcinomas, while the cases of hyperplasias and normal endometrium showed absence or very low p53 expression (< 5% positive epithelial cells). This expression was correlated with histological grade and stage as well as with both proliferative associated indices Ki-67 and PCNA.

pRb

The function of retinoblastoma (Rb) protein is central to cell cycle regulation and causes cell cycle arrest by blocking the E2F family of transcription factors [27]. To our knowledge, there are relatively few studies examining Rb alterations in endometrial lesions. Using an immunohistochemical approach, Rb protein expression was identified in normal, hyperplastic and malignant endometrium [10, 16, 28-30]. Ambros *et al.* [16] found no difference in pRb expression between normal and neoplastic endometrium, while other authors reported an altered expression of the protein especially in high-grade and advanced endometrial tumours [29, 30]. In a previous study, the comparison of normal endometria, hyperplasias and endometrial carcinomas showed that pRb is expressed in all endometrial lesions.

In normal endometria, pRb immunoreactivity was observed predominantly in proliferative endometrium rather than in secretory endometrium and in the majority of proliferating epithelial cells [10]. Similarly, Niemann *et al.* [29] found a strong pRb reaction in all cases of proliferative endometrium, while weak or absent reactivity was observed in secretory endometrium. The authors suggest that this difference of expression is not unexpected since the Rb gene plays a role in the regulation of cell growth and differentiation. It is known that when pRb is phosphorylated, E2F is released and the cell can initiate DNA synthesis by progression of the cell cycle from the G1-phase to the S-phase [27]; it is also well established that, during the proliferative phase of the endometrium, the epithelial cells synthesize DNA. In our previous study [10], no pRb expression was observed in most of the carcinomas, while in the remaining cases moderate or high levels of pRb reactivity were observed. Neither the pRb loss nor the expression of the protein showed any significant correlation with tumour grade or FIGO stage, suggesting that if an alteration of Rb protein occurs, it may be not associated with malignant progression. In addition, in all lesions pRb expression was related to the expression of proliferation indices, suggesting that the Rb gene participates in the growth regulation of endometrial tissue cells.

Cell cycle regulated proteins (cyclin D1, cyclin E, p21/WAF1)

Cyclin D1 and cyclin E: Progression through the eukaryotic cell cycle is mediated both positively and negatively by a variety of growth regulatory proteins. Cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors have important regulatory roles during cell cycle progression. The cyclins are a family of proteins which regulate the progression of cells through the G1-phase of the cell cycle interactions with specific catalytic CDK partners. The D-type/CDK4 complexes can act as protein kinases of pRb in G1-phase [31]. Although there are three D-type cyclins (D1, D2 and D3), there is evidence that cyclin D1 particularly is implicated in human tumorigenesis. Cyclin E is a late G1 cyclin, which, along with its catalytic subunit cdk2, is also involved in the phosphorylation of the Rb protein. The activation of the cyclin E/cdk2 complex is the rate-limiting event for cell transition into the S-phase of the cell cycle [32]. Overexpression of cyclin E accelerates the G1-to S-phase transition and increased expression of cyclin E-related protein has been reported in several human malignancies. Although the role of cyclin D1 in carcinogenesis is not completely understood, several experimental observations suggest that this cyclin plays a key role in the development and progression of the tumours. There are only a few conflicting reports about the immunoexpression of cyclins D1 and E in normal, hyperplastic and neoplastic endometrium [33-39]. Nikaido *et al.* [34] demonstrated that cyclin D1 expression was detected in 30/74 (40%) of endometrial cancer cases and diffuse positivity for cyclin D1 was associated with clinically advanced stage and advanced histological grade. Ito *et al.* [35] found 56% overexpression of cyclin D1 in endometrial carcinoma and they concluded that no significant correlation exists between cyclin D1 immunostaining pattern and survival or the clinical outcome of patients. In our recent study [40] cyclin D1 immunoreactivity was restricted in a few cells of the normal and hyperplastic endometrium. In endometrial carcinoma cyclin D1 was observed in all cases, but only in 18.2% was overexpressed. We found no significant correlation between cyclin D1 immunostaining pattern and clinicopathological parameters.

It has been found that increased cyclin E expression was linked with histological grade in endometrial carcinoma [36, 37]. On the other hand, Ito *et al.* [35] reported that the expression of cyclin E was not correlated with histological grade in endometrial carcinoma. The results of our study [40] showed higher levels of its expression in endometrial carcinoma compared to normal endometrium and endometrial hyperplasia. In addition, cyclin E expression was significantly increased with histological grades of endometrial carcinoma.

p21(waf1/cip1): p21(waf1/cip1) protein belongs to the Kip1/Cip1 family of cyclin-dependent kinase inhibitors. It is a potent negative regulator of the cell cycle that acts as a cyclin-dependent kinase inhibitor mediating p53-induced G1 arrest following DNA damage and maintaining growth arrest in terminally-differentiated cells. In endometrial carcinoma limited studies of immunohistochemical p21 (waf1/cip1) expression have been undertaken [41-46] with conflicting results. Salvesen *et al.* [42] found low p21 (waf1/cip1) expression to be associated with poor histological grade in endometrial carcinomas, while other investigators found no correlation with tumour grade or FIGO stage [41]. In our previous study [46] we found no correlation with tumour grade or FIGO stage. However, a statistically significant positive relationship of p21(waf1/cip1) expression with PR, p53 protein, pRb and the two proliferative associated indices Ki-67 and PCNA was observed. These observations suggest that p21 (waf1/cip1) expression may be involved in cell proliferation and the development of the tumour and could be considered as a marker of poor biological behaviour in endometrial cancer.

Apoptosis-related protein (bcl-2)

Recent advances in molecular biology have shown that alterations in genes controlling proliferation or apoptosis may play a major role in human carcinogenesis.

Bcl-2 is an oncogene involved in the regulation of cell death by inhibiting programmed cell death in physiological and neoplastic conditions [47].

The human endometrium undergoes characteristic proliferative, secretory and menstrual phases as a result of cycle-related changes in the levels of steroid hormones secreted by the ovary. The endometrial breakdown during the menstrual period is related to the apoptotic cell death of endometrial cells [48, 49]. However, little is known about the mol-

ecular mechanisms by which cells of the endometrium undergo cyclic and localized apoptosis. A physiological role of bcl-2 in the regulation of endometrial cell death could suggest that abnormalities in the expression of this gene may play a part in the natural history of endometrial neoplasia [50]. Bcl-2 expression decreased in a group of carcinomas compared with cases of adenomatous hyperplasia, normal proliferative and secretory endometrium. In carcinomas decreased bcl-2 expression was associated with increased tumour grade [51-55].

A relationship between bcl-2 and steroid hormone regulation has been documented in breast carcinomas [54]. Otsuki *et al.* [56] in their study demonstrated the cyclic bcl-2 expression pattern in the normal menstrual cycle, suggesting the regulation of bcl-2 expression by ovarian hormones and specially by the estrogen hormone.

In the our recent study [55] bcl-2 expression was closely correlated with ER and PR immunoreactivity in normal and hyperplastic endometrium, supporting an estrogen-dependent regulation. In addition, bcl-2 expression was positively correlated with PCNA score and pRb expression, suggesting its implication in tumour proliferation.

Adhesion molecule (CD44s)

CD44 is a transmembrane glycoprotein molecule expressed by many normal tissues and is involved in cell-cell and cell-matrix interactions. It is expressed as a standard form – CD44s and as numerous splice variants – CD44v [57]. The cell-to-cell interactions, which are important for basic cellular processes such as proliferation, differentiation, migration, lymphocyte circulation, tumour invasion and metastasis, have roused the interest of several investigators. There are limited and controversial reports about CD44s expression and variant isoforms in endometrial cancer. Saegusa *et al.* [58] reported that the expression of CD44s and CD44v6 was significantly increased in endometrial carcinoma in comparison with hyperplasia and normal proliferative endometrium. Fujita *et al.* [59] reported that the presence of variant forms of CD44 was significantly higher in normal endometrium (81.8%) and decreased in hyperplasia cases (42.9%), and the lowest expression was observed in cancer cases (17%). It has been found that CD44 variants 6 and 7 expressions were correlated with tumour grade [60, 61]. In addition, decreased CD44v6 expression was associated with myometrial invasion [62, 63]. Stokes and co-workers [63] showed only a trend of CD44s-positive staining in non-invasive tumours versus invasive and non-invasive/superficially invasive, versus deeply invasive tumours. In our recent study [64] we found no relationship of CD44s expression with tumour grade, but we found a decreased CD44s expression to be associated with myometrial invasion. The transmembrane receptor protein CD44 is known to belong to the family of adhesion molecules, which is involved in cell-cell and cell-matrix interactions. Thus, decreased CD44s expression may indicate an implication in tumour progression.

There are conflicting reports on endometrial carcinoma regarding CD44 variant expression and a tendency to invade the lymph-vascular space. Some investigators reported that the risk of lymph-vascular space invasion was less in CD44v6- expressing tumours [65, 66]. However, Yorishima and co-workers [67] reported that there was a linear correlation between CD44v6 expression and lymph-vascular space invasion. In our study we found no relationship between CD44s expression with lymph-vascular space invasion. These discrepancies could be related to different technical methods or differences in the interpretation of the results or even differences in the patient population.

Cathepsin D

The ability of tumour cells to invade tissues and metastasize is thought to involve an increased expression of proteases and a decrease in the levels of protease inhibitors. Proteases may facilitate metastasis in different ways including detachment of individual cells from the primary tumour, invasion of surrounding tissues to allow contact with vascular channels, degradation of the basement membrane during both intravasation and extravasation and invasion of tissues during formation of secondary tumour sites. Degradation of the basement membrane structures by proteases is one of the initial events in the process of invasion by carcinoma cells and several classes of proteases have been implicated in this process [68]. Cathepsin D (CD) is an aspartyl lysosomal protease, which is widely expressed in all cells throughout the body. The role of CD in tumour progression has come from in vitro and in vivo studies. In vitro, CD has the ability to digest extracellular matrix, including basement membrane components [69]. It also has mitogenic properties [69] and facilitates the release of an angiogenic factor, basic fibroblast growth factor (bFGF) stimulating neovascularization [70]. In addition, CD has been shown to be inducible by estrogens [71] and has therefore been extensively studied in breast carcinomas [72-74]. However, in carcinomas of the endometrium and in other types of carcinomas [75-77] it has not yet been established as a prognostic marker. To our knowledge, there are at present few reports investigating the prognostic value of CD in endometrial lesion by immunoradiometric assay [78-81] and even fewer concerning the expression of this enzyme by immunohistochemical methods [82-85] or both [86]. A gradually decreasing CD expression in cancerous and hyperplastic endometrium compared to normal endometrium using an immunoenzymatic assay was found [78]. In carcinomas, a relationship of CD expression with tumour stage was also detected [79, 81, 84]. Other studies on tumour cytosol CD level or immunohistochemical CD expression found a correlation with tumour grade [79, 85]. In a recent study we found no relationship between CD expression and tumour grade, stage or hormone receptor status [87]. There are conflicting results about the correlation of CD expression of endometrial carcinomas with hormone receptor status. Some investigators found no relationship of CD with hormones receptor status by immunoradiometric assay [79, 82] or by immunohistochemical methods [86], in contrast to the findings of other investigators [81].

Other markers (hsp27, metallothionein)

Hsp27: Self-protection and survival characterize the cells of all organisms, which respond with an intrinsic mechanism to heat shock by activating genes coding for the heat shock proteins (hsps). Besides heat shock, other environmental stresses such as exposure to heavy metals or oxidants and physiological stresses, ischemia, viral and microbial infections, as well as inflammation have been mentioned as being responsible for the synthesis of hsps. The different levels of hsp induction depend on the organism, cell type, cellular conditions, type and intensity of the stimulation [88]. Hsp27 is a member of the heat shock protein family, with low molecular weight. Initially it was named 24-kDa protein and was found in human MCF-7 cells and human breast cancer cell lines [89]. It was characterized as an estrogen-regulated protein because of its expression in several hormone-sensitive organs [90, 91]. In human tumours the role of hsp27 is unknown and there are controversial results about its prognostic contribution. In endometrial carcinoma, a limited number of studies have been performed aiming to explore the biological significance of hsp27 [92-98]. Hsp27 expression was found to be higher in endometrial lesions, non- or minimally invasive lesions and early stage cancers [96]. Lower levels of hsp27 were found in patients who had tumour recurrences and in patients who died from the disease [94, 95]. Furthermore, its expression was characterized as an independent prognostic indicator in patients with endometrial carcinoma [96]. The results of our previous study showed lower hsp27 expression in carcinomas compared with hyperplastic and normal endometrium suggesting that in endometrial carcinomas the absence or the lower expression of hsp27 may creat a defective mechanism against stressful conditions [11]. Hsp27 has been shown to be directly related to estrogen receptor status in endometrial carcinomas according to the results of other studies [97, 98]. It has been shown that hsp27 is estrogen-inducible in estrogen-dependent cell lines [99, 100]. In addition, endometriod tumours showed significantly higher hsp27 immunostaining than non-endometriod [97]. Paradoxically, hsp27 expression did not correlate with ER status in hyperplastic and normal proliferative endometrium, while a positive relationship of its expression with ER status was found in normal secretory endometrium and in the group of carcinomas. In hyperplastic and normal proliferative endometrium, where the estrogen receptor status is at a high level, hsp27 expression is unrelated to these receptors. The results of the present study also showed a positive relationship of hsp27 expression with PR status in endometrial carcinomas in contrast to the findings of other investigators [98], suggesting that hsp27 may be regulated by the synergistic action of estrogen and progesterone. A statistically important correlation was noticed between hsp27 expression and premenopausal status; the expression decreased with age and can be explained by estrogen status which is more activated in premenopausal women. In this study we failed to demonstrate a correlation between hsp27 expression and tumour grade or stage. However, other investigators have shown increased hsp27 expression to be correlated with increased tumor cell differentiation [94-96]. Furthermore, it has been shown that hsp27 is also involved in cell proliferation and several studies correlated its expression with proliferative associated indices. Khalid *et al.* [101] showed a positive relationship of hsp27 expression with Ki-67 labeling index in human astrocytomas in contrast to the findings of other investigators in other types of tumours [102]. In addition, no correlation of hsp27 expression with p53 was found [102] which is in accordance with our results. Vargas-Roig *et al.* [103] found an inverse relationship of hsp27 expression with PCNA proliferating index in breast carcinomas, and this finding added evidence to the concept that hsp27 may be involved in cell growth arrest. In our study [11] we did not find such a correlation, perhaps suggesting different pathways of cell proliferation.

Metallothionein: Metallothioneins (MTs) represent a group of low-molecular-weight cystein-rich intracellular proteins that act to bind and detoxify group IIb heavy metal ions [104, 105]. The synthesis of metallothionein (MT) is induced in a variety of tissues by these metal ions as well as by endogenous factors such as glycocorticoids, interferon, interleukin-1 and vitamin D [106, 107]. Furthermore, MTs are implicated in a transient response to any form of stress or injury, providing a cytoprotective mechanism against the potentially damaging effects of oxygen-derived free radicals [104]. Our knowledge of MT expression in human tumours is limited and there are at present few reports investigating the expression of MT in endometrial lesions [108]. The results of our study showed that MT values were higher in the secretory phase and decreased in the proliferative phase as well as in the cases of hyperplasia [109]. In the latter, MT expression was usually detected in foci with atypical features in which the process of malignant transformation might initiated. Therefore, MT expression could be considered as an early event of endometrial carcinogenesis. In addition, MT expression was positively correlated with tumour grade, with the proliferative index Ki-67 as well as with p53 expression in the case of carcinomas. An inverse correlation of MT expression with PR content was also found. Our results suggest that MT expression is correlated with the more aggressive phenotype of endometrial carcinoma according to the other types of malignancies [108, 110-115]. We did not find any statistically significant difference in MT expression with tumour stage, in contrast to the finding of McCluggage *et al.* [108].

Conclusion

The immunohistochemical expression of the biomarkers studied seem to contribute to endometrial carcinogenesis in a hormone-dependent or independent manner. The biomarkers Ki-67, CD44, hsp27 and MT appear to be under hormonal control. The biomarkers bcl-2, Rb and CD seem to contribute to endometrial carcinogenesis as an early event, while c-erbB-2, p53, p21 and MT take part in the late stage of the disease. The biomarkers Ki-67, PCNA, p53, c-erbB-

2, cyclin E and p21/waf1 appear to be correlated with tumour differentiation and can be considered as indicators of the biological behaviour of endometrial carcinoma. CD44 is correlated with tumour stage and can be considered as an indicator of progression of the disease. The interrelationships of biomarkers show that these genetic dysregulations can be implicated in the control of cell proliferation and through these pathways might contribute to the multistep process of endometrial carcinogenesis.

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Address reprint requests to:
E. IOACHIM, M.D.
Department of Pathology,
Medical School,
University of Ioannina
45110 Ioannina (Greece)