

New Biomarkers for Cervical Cancer – Perspectives from the IGF System

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1. Introduction

The insulin-like growth factor (IGF) family is organized in a complex regulatory network at the cellular and sub-cellular levels. In the human the IGF system has a key physiological role in the development of the organism and maintenance of normal cellular function during foetal and postnatal life. The IGF system consists of three ligands, IGF-I, IGF-II and Insulin; three cell membrane receptors, IGF-I receptor (IGF-IR), insulin receptor (IR), and IGF-II receptor (IGF-IIR); six high-affinity IGF binding proteins, IGFBP-1 through -6, their specific proteases (IGFBP proteases) and membrane receptors (IGFBP-R) (Fig. 1).

IGF-I and IGF-II share a 62% homology in amino acid sequence and there is a 40% homology between the IGFs and proinsulin (Furstenberger & Senn, 2002). IGF-IR is a member of the family of the tyrosine kinase growth factor receptors and is highly homologous at the amino acid sequence level to the IR. The mature membrane receptor is a tetramer made of two α -chains and two β -chains, with several disulfide bridges (LeRoith et al., 1995). The extracellular α -subunits form the ligand binding domain and several lines of evidence suggest that the binding sites for IGF-I and IGF-II may be distinct (Samani et al., 2007). IGF-I and IGF-II bind to IGF-IR with high affinity, however, ligand affinities may vary with cell type and experimental conditions. IGF-II can also bind to the insulin receptor isoform A (IR-A) with an affinity similar to that of insulin. IR-A is expressed in certain tumours and has a more mitogenic effect than the IR-B isoform, the latter having a more metabolic function (Pandini et al., 2002). In cells expressing both IR and IGF-IR, IR hemireceptors may heterodimerize with IGF-IR hemireceptors, leading to the formation of hybrid receptors (IR/IGF-IR). The proportion of hybrid receptors is a function of the mole fractions of each receptor. Early studies carried out with purified hybrid receptors indicate that these receptors mostly bind IGF-I and that they bind insulin with a much lower affinity (Belfiore et al., 2009).

IGF-II can also bind to a second receptor, IGF-IIR, which is a multifunctional single transmembrane glycoprotein, identical to the cation-independent mannose 6-phosphate receptor. It is composed of a large extracytoplasmic domain and a short cytoplasmic tail that lacks intrinsic cytoplasmic activity (El-Shewy & Luttrell, 2009). IGF-IIR perform diverse

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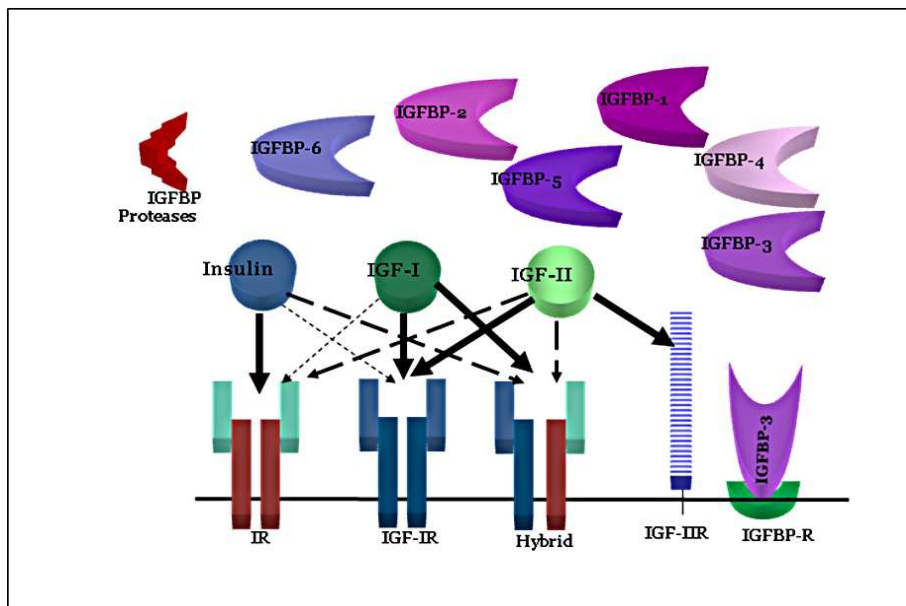


Fig. 1. The IGF system. The IGF system consists of the receptors (IR, IGF-IR, IGF-IR/IR hybrids and IGF-IIR), the peptides (IGF-I, IGF-II and insulin), six high affinity IGFBPs (-1 to -6), IGFBP proteases and IGFBP receptors (IGFBP-R).

cellular functions related to lysosome biogenesis and the regulation of growth and development. The IGF-IIR receptors recycle continuously between two cellular pools and at steady state most of the receptor localize in endosomes and the remainder (approx. 10%) on the plasma membrane. Traditionally, the IGF-IIR was considered a scavenger receptor, regulating the extracellular IGF-II concentrations, but recent studies suggest that the IGF-IIR also functions in signal transduction and may play an important role in tumour progression (El-Shewy et al., 2006).

To add more complexity to the system, the IGFs in circulation and in the tissues, are associated with a family of six high affinity IGF-binding proteins (IGFBPs) that regulate the bioavailability of the IGFs (Baxter, 2000). The IGFBPs regulate the interaction of IGFs with the receptors. In human about 80% of circulating IGF-I is carried by IGFBP-3/acid label subunit complex (Lewitt et., 1994). All IGFBPs inhibit IGF action by sequestering IGFs and some IGFBPs (IGFBP-1, -3, -5) can also potentiate IGF action. The resulting change in the ratio of IGF to IGFBP modulates IGF/IGFBP/IGF receptor interactions and may play a role in normal and abnormal tissue proliferation (Mohan & Baylink, 2002).

Overexpression of growth factors and/or their receptors is a common event in malignancy and provides the underlying mechanism for uncontrolled proliferation, one of the hallmarks of cancer (Hanahan & Weinberg, 2000). The IGF signalling system network is tightly controlled under normal physiological conditions and alterations that disrupt the delicate balance of the system can trigger a number of molecular events that can lead to malignancy. Many studies have involved the IGF system in carcinogenesis and tumour progression of

different cell types (LeRoith & Roberts, 2003). Many cancers have been shown to overexpress the IGF-I receptor and/or the ligands (IGF-I and IGF-II) and some combinations of the six IGF binding proteins (IGFBPs) (Samani et al., 2007). It appears that abnormal IGF-IR activation can result in oncogene activation leading to increased cellular proliferation and malignancy. Although the IGFs are not in themselves tumorigenic factors, it has been shown that overexpression of IGF-II, as in the case of loss of imprinting (LOI) (Pavelic et al., 2002), can contribute to gynaecological malignancies like ovarian cancer (Murphy et al., 2006) and choriocarcinoma (Diaz et al., 2007).

Compared to other types of cancer, like breast, colon, prostate, and lung, little research has been done on the relationship between the IGF family and cervical neoplasias, and by now, much remains to be learned. In this review, we will discuss some of the studies focused on the role of the components of the IGF system in the progression of cervical cancer and its potential utility as a diagnostic biomarker.

2. The IGF system and cervical cancer

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in females worldwide, accounting for 9% (529,800) of the total new cancer cases and 8% (275,100) of the total cancer deaths among females in 2008. More than 85% of these cases and deaths occur in developing countries. Worldwide, the highest incidence rates are in Eastern, Western and Southern Africa, as well as South-Central Asia and South America (Jemal et al., 2011). In Colombia, cervical cancer is one of the most common causes of cancer mortality among women (Ferlay, 2010).

Persistent infection with high-risk types of HPV (HR HPV) has been identified as the main risk factor for the development of cervical cancer and its precursor lesions, squamous intraepithelial lesions (SIL) (Walboomers et al., 1999; Muñoz et al., 2003). SILs precede the development of cervical cancer and are classified in two groups: low-grade SIL (LSIL), and high-grade SIL (HSIL) (Solomon et al., 2002) (Fig. 2). Although HPV infections are among the most frequent sexually transmitted diseases, infections are usually self-limited and revert spontaneously, with only a small group of women developing cervical cancer (Woodman et al., 2001). The evolution of infection to LSIL, HSIL and cancer is dependent on several factors, many of which remain to be identified. Despite intensive investigation, the tumor biology of this disease is still largely unknown. Although prognostic factors such as pelvic lymph node metastasis affects the outcome of cervical cancer, the variability in progression-free and overall survival (OS) among patients with similar clinical and pathological characteristics, makes it difficult to predict the outcome reliably (Huang et al., 2008). Current research strives to determine why certain HPV-positive women develop cervical cancer while others do not (Schaffer et al., 2007).

2.1 Serum levels of IGFs as cervical cancer biomarkers

IGF-I is a potent mitogenic growth factor that plays a critical role during embryogenesis and development in human and animal species. Together with Growth Hormone (GH) constitute an axis that regulate postnatal growth and development in an endocrine, paracrine and autocrine mode of action. IGF-I is produced by numerous adult organs, with major contribution of liver to overall circulating IGF-I levels. After puberty, circulating IGF-I

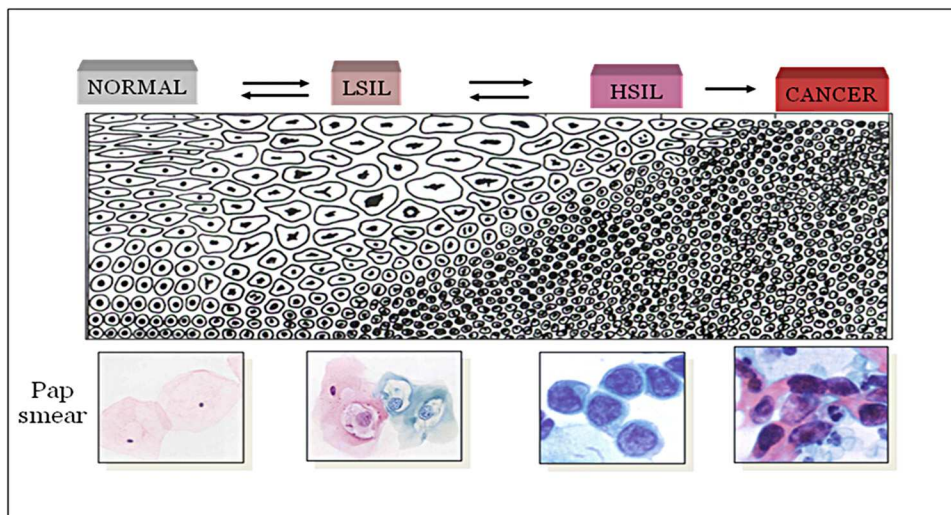


Fig. 2. Schematic representation of cervical cancer and its precursor lesions. Low-grade squamous intraepithelial lesions (LSIL), High-grade squamous intraepithelial lesions (HSIL).

levels decline, in contrast to IGF-II levels that remain elevated throughout adult life (Bang & Hall, 1992).

The proliferative and anti-apoptotic effects of IGF-I observed in animal and cell cultures, made it an obvious risk factor candidate in cancer development. In attempt to find a reliable serum biomarker to predict occurrence, progression or prognosis of human cancer, scientists have investigated the correlation of serum IGF-I or IGFBP-3, the most abundant IGFBP in circulation, to the prevalence of a variety of cancers. Prospective and retrospective studies have demonstrated an association between high concentrations of serum IGF-I and increased risk for prostate and premenopausal breast cancer (Renehan et al., 2004), whereas an inverse association has been reported for certain types of cancer, including gastric (Lee et al., 1997), endometrial (Lacey et al., 2004), liver (Stuver et al., 2000), and contradictory results for lung cancer (Yu et al, 1999; Mazzoccoli et al., 1999; Renehan, 2004).

With respect to cervical cancer, results have not shown consistency. Mathur et al. found that serum IGF-II levels were elevated, whereas IGFBP-3 levels were decreased, across the cervical lesion spectrum, and proposed that this could be used as an aid for early diagnosis and predicting prognosis of cervical cancer (Mathur et al., 2000, 2003, 2005). Two other epidemiological studies (Lee et al., 2010; Wu et al., 2003) did not find an association between IGFBP-3 levels and risk of squamous intraepithelial lesions, but on the other hand, a strong significant increase in the risk of low-grade or high-grade squamous intraepithelial lesions was observed for women with IGF-I serum levels in the highest versus the lowest quartiles.

We conducted a case-control study on Colombian women with SIL and cervical cancer, and our findings illustrated an inverse association between cervical cancer risk and IGF-I circulating levels. In accordance to this result, IGF-I/IGFBP-3 molar ratio was also inversely associated with cervical carcinoma (Serrano et al., 2006). Similar results were obtained in a

study conducted in patients with diagnosed pre-cancer lesions where levels of IGF-I were inversely associated with risk of high-grade cervical intraepithelial neoplasia (Schaffer et al, 2007).

A recent prospective study reported a possible influence of the IGF axis on the natural history of oncogenic HPV and the development of cervical neoplasia (Harris et al., 2008). A high IGF-I/IGFBP-3 ratio was associated with increased persistence of oncogenic HPV infection [adjusted hazard ratio (AHR), 0.14; 95% confidence interval (95% CI), 0.04-0.57], whereas IGFBP-3 was inversely associated with both the incident detection of oncogenic HPV (AHR, 0.35; 95%CI, 0.13-0.93) and the incidence of oncogenic HPV positive cervical neoplasia (that is, squamous intraepithelial lesions at risk of progression; AHR, 0.07; 95% CI, 0.01-0.66). We conducted a case-control study with in a prospective populational-based cohort of 2200 women, followed-up during 10 years. Results adjusted by age, menarche, smoking, parity and hormonal contraceptives, showed that high IGF-I serum levels were associated with persistence (lower vs. higher quartile: RR 2.60 95% CI 0.61-10.94) and prevalent (lower vs. higher quartile: RR 2.67, 95% CI 0.37-19.09) HPV infection, however, no significance was achieved, and no association can be demonstrated (Serrano et al., 2010b).

A recent nested case-control study (151 cervical cancer cases, 443 controls), found an inverse correlation between IGFBP-3 serum levels in pregnant women and risk of cervical cancer [OR 0.43 (95% CI 0.21-0.86)], suggesting that IGFBP-3 measured in pregnancy may be a marker of lower risk of cervical cancer (Jeffreys et al., 2011). In early-stage cervical cancer patients, lower IGF-I levels seemed to be associated with worse overall survival rate, but was not of an independent value, and there was no relationship between IGFBP-3 levels and survival (Huang et al., 2008).

It is unclear why increased serum IGF-I levels may have a protective effect on development of cervical cancer, whereas in breast and prostate cancer it has the opposite effect. One of the theories is that the natural history to cervical cancer, where infection with HPV plays a crucial role, differs from sex hormone-related cancers (Schaffer et al., 2007). Larger prospective investigations are indicated to better clarify these associations, including any potential HPV type-specific differences in the effects of IGFs.

2.2 Aberrant expression of IGFs in cancer

Several lines of evidence support an important role for the IGF system in tumor tissue. IGF-IR activation is involved in several processes associated to oncogenic transformation, such as proliferation, migration and invasion (Coppola et al, 1994; Pavelic et al., 2002; Samani et al., 2007; Sell et al., 1994). IGF-I expression rarely occurs in tumoral cells, but can be produced by stromal cells surrounding the tumor. IGF-II, IGF-IR and certain IGFBPs are overexpressed in many primary tumors and cancer cell lines (Cullen et al., 1990; Hellawell et al., 2002; Werner & LeRoith, 1996). IGFBP-3 generally has, with some exceptions, a rather inhibiting effect on IGF action in tumor tissues (LeRoith & Roberts, 2003).

Increased expression of IGF-I, IGF-II, IGF-IR, or combinations thereof have been documented in various malignancies including glioblastomas, neuroblastomas, meningiomas, medulloblastomas, carcinomas of the breast, malignancies of the gastrointestinal tract, such as colorectal and pancreatic carcinomas, and ovarian cancer (Samani et al., 2007). These data show that whereas a correlation between IGF-I/ IGF-II

expression levels and tumor progression could be consistently documented in some malignancies (e.g., colorectal, hepatocellular, and pancreatic carcinomas), no consistent correlation was seen in others (e.g., breast cancer). Moreover, in some cases, conflicting results were obtained in different studies that analyzed the same types of cancers (e.g., gliomas) (Samani et al., 2007). Overexpression of IGF-II has been reported in several malignancies. A study identified the loss of imprinting (LOI) of the gene, frequently observed in the colonic mucosa of colorectal carcinoma patients, as a risk factor for developing colorectal carcinoma (Cui et al., 2003). Overexpression of IGF-II and IGF-IR, was observed in gestational trophoblastic neoplasias, including hydatidiform moles and choriocarcinoma, correlating with the elevated IGF-II levels in the circulation (Díaz et al., 2007). Taken as a whole, these studies suggest that the IGFs can play a paracrine and/or autocrine role in promoting tumor growth.

IGF-IR may also form hybrid receptors with one α - and β - subunit from the IR. There are two IR isoforms formed by alternative splicing of exon 11, IR-A which lacks exon 11 and IR-B which contains exon 11. IGF-I and IGF-II, bind to hybrid receptors IGF-IR/IR-A leading to mitogenic signaling. IGF-II and insulin bind to IR-A leading also to mitogenic signaling, whereas, activation of IR-B by insulin, or the hybrid IGF-IR/IR-B by IGF-I, results mostly in metabolic signaling. After *in vitro* and *in vivo* studies demonstrating that insulin may also play a significant and independent role in tumorigenesis, insulin is now receiving more attention in this regard (Gallagher & LeRoith, 2011).

In the past few decades, accumulating evidence has established that insulin receptors (IRs) are usually abnormally expressed in cancer cells, where they mediate both the metabolic and nonmetabolic effects of insulin. Most recently, it was observed that splicing of the IR gene is altered in cancer cells, thus increasing IR-A/IR-B ratio, which affects the cell response to circulating IGFs and insulin (Belfiore et al., 2009). As it was mentioned earlier, many tumors overexpress IGF-II, which also signals through the IR-A isoform. An important role of the IGF-II/IR-A loop has been observed in gestational trophoblastic diseases. Both IGF-I and IGF-II stimulated JEG-3 choriocarcinoma cell invasion, although they signal through different receptors: IGF-I through IGF-IR, and IGF-II through IR-A. In JEG-3 cells, which predominantly express IR-A, IGF-II stimulated cell invasion more potently than insulin (Díaz et al., 2007). The effects of IGF-II may also be mediated by the IGF-IIR, as reported in trophoblast cells (MacKinnon et al., 2001), giving more relevance to the role of this receptor in signaling and tumorigenesis.

2.2.1 Tissue expression of IGFs as risk or prognosis biomarkers in cervical cancer

In a recent study the expression levels and activated status of IGF-IR were measured by immunohistochemistry in formalin-fixed and paraffin-embedded specimens. IGF-IR levels and phosphorylation status were significantly high in cervical intraepithelial neoplasia (CIN III) and invasive cancer specimens (Kuramoto et al., 2008). An interesting retrospective analysis of patients with early-stage cervical cancer evaluated IGF-IR expression levels by immunofluorescent stain. Authors found that high-grade expression of IGF-IR, is an independent predictor of cervical cancer death and recurrence, and when combined with elevated squamous cervical cancer antigen (SCC Ag) serum level, could further help identify the subgroup of patients at higher death risk. Colocalisation of IGF-I and IGF-IR in the cancerous tissues, and the lack of correlation between circulating IGF-I or IGFBP-3, and

IGF-IR overexpression in cervical cancer tissue, give support to a paracrine or autocrine function of the IGF system in early-stage cervical cancer, with the corresponding adverse IGF-I stimulation of IGF-IR signaling (Huang et al., 2008).

Only few studies have addressed the analysis of gene expression on exfoliated cells. The remaining cellular material after preparation of routine Pap smear can be used for the search of new biomarkers at both the mRNA and protein levels. The use of cervical scrapes instead of biopsies for biomarker studies has several advantages, since this is a noninvasive procedure, the material obtained is not affected by stroma or other contaminants, as is the case in the analysis of tissue homogenates and can be the base for molecular epidemiology studies.

In a study on cervical scrapes from SIL and cancer patients, we found no difference in IGF-II mRNA levels between cancer and normal cells, whereas at the protein level, IGF-II expression was reduced in cancer cervical scrapes (Serrano et al., 2007, 2010a). There is ample evidence that the levels of IGF-II mRNA differ from the protein. While IGF-II transcripts were higher than in normal tissue in prostate cancer (Tennant et al., 1996), Wilms tumors (Haselbacher et al., 1987), and glioblastomas and astrocytomas (Hultberg et al., 1993), it was not the case for the protein. High levels of IGF-II protein, but not mRNA were found in pheochromocytomas (Haselbacher et al., 1987). These evidences indicate that regulation of IGF-II expression involves transcriptional mechanisms, as genomic imprinting, but also post-transcriptional regulation, that can occur through malignant progression. A member of the IGF-II mRNA binding protein family (IMP), IMP-3, has been reported to be an activator of IGF-II mRNA translation and therefore may play a critical role in IGF-II-dependent cellular proliferation (Liao et al., 2005). IMPs were detected in various cancers; increased levels were found in ovarian cancer and correlated with prognosis. There are no studies about IMP in cervical cancer.

Few studies have examined the expression of hybrid receptors in cervical cancer cells. In a recent study, we found that IR-A, IR-B and hybrid receptors coexist with IGF-IR in human papillomavirus-positive cervical cancer cells. Tyrosine phosphorylation of the receptors and activation of the MAPK and PI3-K pathways were observed upon ligand binding, which may explain the anti-apoptotic effect mediated by IGF-I in these cells (Serrano et al., 2008). Further studies are required to fully understand the significance of the hybrid receptors, especially IGF-IR/HR-A in cervical carcinogenesis and for the design of therapies to target their effects.

2.2.2 The role of IGF in radioresistance in cervical cancer

The crucial role of IGF-IR in promoting resistance to cancer treatments, such as radiation and chemotherapy, is well documented for several cancer types (Samani, et al., 2007). IGF-IR down-regulation by antisense nucleotides or its inhibition by tyrosine kinase inhibitors increases cancer cell sensitivity to both radiation and chemotherapy in a variety of malignancies. Some studies indicate an important role of IGF-IR in several pathways involved in the induction of radioresistance (Belfiore et al., 2009).

The identification of the mechanisms underlying the effects of IGF-II/IGF-IR signaling in the induction of resistance to oxidative stress in cancer cells may provide useful information for the selection of the appropriate treatment strategy. In a study with advanced cervical

cancer patients (CIN I-III), we found that tumor tissues co-expressing IGF-IR and IGF-II, showed an increase (4.6-fold) in the risk of developing resistance to radiotherapy. The increased expression of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in these cancer tissues, demonstrated the metabolic shift to glycolysis under hypoxic conditions (Moreno-Acosta et al., 2010). Therefore, targeting IGF-II/IGF-IR signaling may be a desirable option in the treatment of tumors overexpressing the IGF-IR.

3. Biomarkers for cervical cancer

Biomarkers used for screening in cancer, usually consist of biomolecules that are released from tumors or precancerous lesions into the bloodstream, urine, or other means, they should have the property to survive the metabolic degradation to be measurable (Ahlquist, 2010). Molecular changes that occur in the development of tumors may take several years; some biomarkers can be used to detect early stage disease. However, biomarkers are usually found at relatively low level compared to other biomolecules; research and validation as possible diagnostic tests depend critically on the ability to achieve measurements with high precision and sensitivity.

Several biologic markers or indexes have been studied as potential tools to determine the prognosis and biological behaviour of various types of gynaecological neoplasias. From years the search of cervical cancer biomarkers has been fed through different techniques, such as immunohistochemistry (IHC), probably the most affordable and simple technology to detect many such biomarkers (McCluggage, 2002; Munoz et al., 2003). This fact prompted investigators to develop and test antibodies in a widespread range of neoplastic lesions. In this approach, some researchers found a two component system, denominated MaTu, consisting of an exogenous transmissible MX agent (coding for p58X protein) and an endogenous MN (coding for MN-protein) in HeLa cells (Závada et al., 1993). The association between MN protein (MN-p) expression and oncogenesis was suggested by its presence in neoplasms, and not in benign tissue (Brewer et al., 1996; Liao et al., 1994). Because HeLa is a cultured human uterine cervix carcinoma cell line, these authors concluded that MN-p had great use as a tumour marker in the evaluation of cervical carcinoma and could predict progression in precursor cervical intraepithelial neoplastic (CIN) lesions. Nonetheless, its utility was questioned as MN-p immunostaining was not limited to neoplastic tissue, but found in adjacent basal cell hyperplasia, metaplasias and benign glandular epithelium (Liao et al., 1994; Resnick et al., 1996).

Ki67, a nuclear proliferation associated antigen is expressed in the growth and synthesis phases of the cell cycle (G1, S, G2, and mitosis) but not in the resting phase, G0. The proportion of cells which express Ki67 (Ki67 index) is an excellent measure of neoplasm proliferation. Production of specific antibodies against recombinant DNA Ki67 antigen (MIB-1) confirmed the definite use of MIB-1 immunoreactivity, exhibiting close correlation with CIN grade and providing additional risk prediction beyond HPV type (Resnick et al., 1996). In low-grade CIN, anti-MIB-1 staining is not detected in superficial levels of epithelium, whereas in high-grade CIN full thickness staining is present. The authors' hypothesis was that viral gene expression undergoes programmed cessation in superficial layers of low-grade CIN; therefore viral E6/E7 protein is no longer present to inhibit p53 and the cells stop cycling (so Ki67 detection through anti-MIB-1 disappears). In high-grade CIN, viral expression of E6/E7 proceeds unabated often in absence of vegetative viral

functions leading to inhibition of p53 and uninhibited cell cycling (so Ki67 detection through anti-MIB-1 continues). In contrast with MN-p expression, anti-MIB-1 shows consistent results by most investigators and exhibits definitive, important associations with clinical outcome and basic carcinogenesis hypotheses in the uterine cervix (Costa, 1996).

For several years, it has been known that high vascularity is characteristic of grade 3 CIN and invasive lesions, and angiogenesis has been associated as indicator of prognosis. The onset of angiogenesis in human cervical cancer occurring during the premalignant dysplastic stage is unique. An association between lymphatic high microvessel density with vascular invasion and a lower global survival rate has been described. There is a study demonstrating that microvessel density in carcinomas of the uterine cervix is a factor associated with poor prognosis (Tjalma et al., 1999). The authors showed that during the progression from noninvasive to microinvasive cervical carcinoma, the microvessel density increases significantly. However, the vessel density does not predict recurrence of noninvasive lesions. Once the tumour cells have passed the basal membrane, the tumour apparently switches to an angiogenic phenotype. Angiogenesis becomes a tumour initiator with prognostic potential (Tjalma et al., 1999). Measures of factor VIII, CD31, and vimentin, showed no association between microvessel count and lymph node invasion, depth of invasion, and histological differentiation. Nevertheless, these studies found an association between lymphatic vessel invasion and a larger number of newly formed vessels (Di Leo et al., 1998).

Other studies have proposed anti-CD34 as a marker for evaluating angiogenesis in cervical cancer (Vieira et al., 2004, 2005). Anti-CD34 antibody is a highly sensitive marker for endothelial cell differentiation and has also been studied as a marker for vascular tumours (Stross et al., 1989; Traweek et al., 1991). Vieira et al. (Vieira et al., 2005) showed that this antibody was able to detect a number of microvessels on neoplasm of the uterine cervix better than anti-factor VIII. They suggested that anti-CD34 antibody reactivity in cervical carcinoma is associated with pathoanatomical features, as microvessel density or invasion, indicative of poorer prognosis and greater risk of recurrence (Vieira et al., 2005). Briefly, they showed lymphatic invasion in 40% of the cases, while vascular and perineural invasion was observed in 24% and 19% of the cases, in a population of 62 patients diagnosed with invasive cervical carcinoma, stages Ib and IIa. They also observed that 55% of women whose carcinoma presented high microvessel density had an undifferentiated type of cancer, suggesting that the presence of lymphatic invasion may associate with higher microvessel density. In general, different markers for angiogenesis may or may not present a statistically significant association with different pathoanatomic features of cervical cancer. These possible markers should be tested prospectively as a prognostic and predictive factor of the response to different therapies used in cervical carcinoma.

On the other hand, a recent study explored the power of serum markers such as squamous cell carcinoma antigen (SCC), CYFRA 21-1, CA 125, immunosuppressive acidic protein (IAP) and vascular endothelial growth factor (VEGF) in patients with cervical cancer (Gadducci et al., 2008). Squamous cell carcinoma antigen comprises two similar proteins, SCC-1 and SCC-2, that possess protease inhibitory property: SCC-1 inhibits chymotrypsin and cathepsin L, while SCC-2 inhibits cathepsin G and mast cell chymase. Both proteins exert anti-apoptotic effects. The mechanism of protection of tumour cells from apoptosis involves the inhibition of caspase-3 activity and/or upstream proteases. SCC-1 and SCC-2

reside in the cytosol of squamous cells, and their presence in the sera of patients with advanced squamous cell carcinomas is mainly due to a passive release rather than an active secretory process into the circulation. Increased SCC levels have been observed in 28-88% of patients with squamous cell cervical cancer. Pre-treatment SCC levels reflect tumour stage and size, cervical stoma invasion, lymph-vascular space status, parametrial involvement and lymph node status; however, its clinical relevance is still debated.

CA 125, an antigenic determinant of a high molecular weight glycoprotein, is recognized by a monoclonal antibody developed against a human ovarian cancer cell line. Elevated CA 125 levels are detectable in 20-75% of patients with cervical adenocarcinoma and have been associated with advanced tumour stage, large tumour size, high histological grade, lymph node involvement and status. CA 125 is detected in normal adult fallopian tube, endometrium, endocervix and peritoneum, and *in vitro* studies showed that CA 125 secretion by human mesothelial cell monolayers may be enhanced by the inflammatory cytokines interleukin-1 and tumour necrosis factor. However, the specificity of the antigen is not yet optimal, since elevated serum CA 125 can be found in benign gynaecological conditions, such as endometriosis and pelvic inflammatory disease, benign non-gynaecological conditions, such as hepatitis, pancreatitis, renal failure and pleural effusion and non-gynaecological malignancies, including lung cancer, pancreatic cancer and non-Hodgkin's lymphoma.

Serum levels of vascular endothelial factor (VEGF) are often elevated in patients with cervical cancer, and decrease significantly after successful treatment. However, the clinical relevance of serum VEGF is still investigational (Gadducci et al., 2008).

New predictive biomarkers for cervical cancer are still necessary to improve the accuracy of screening and thereby reduce overtreatment and possibly improve cost-effectiveness of the treatment. Advances in molecular biology and high throughput technologies have heralded a new era in identification of biomarkers and molecular targets related to carcinogenesis to improve our understanding of the disease and will facilitate screening, early detection, management, and individualized targeted therapy.

3.1 Proteomic studies

Proteomics has emerged as a promising tool for unravelling protein signatures that are associated with a particular malignancy that can be useful biomarkers of the disease. High-throughput technologies like surface-enhanced laser desorption and ionization-time of flight mass spectrometry (SELDI-TOF MS), nanoHPLC-MS/MS or the combination of one- or two-dimensional gel electrophoresis (1-DE, 2-DE) methods with multidimensional chromatographic separation and MS provide a snapshot of the proteome. Validated protein biomarkers could be useful in early detection of disease, monitoring disease progression or monitoring response to treatment.

3.1.1 Proteomic studies on body fluids

After exploring markers in tissue, several studies have focused on biomarker discovery in body fluids which would be advantageous for eventual clinical implementation. Cervical mucous or cervical vaginal fluid (CVF) is potentially an ideal sample to screen for biomarkers for early detection of cervical cancer. As cervical mucous is produced in the

microenvironment where cervical neoplasia arises, it is likely to include proteins produced by the lesion as well as by the host in response to the lesion. Several studies have analyzed peptides and proteins present in human cervical mucous (Dasari et al., 2007; Pereira et al., 2007; Tang et al., 2007). A recent study identified 151 new proteins that included proteins present in the lower female genital tract, such as HBD-2 and cathelicidin, two proteins that play an important role in the innate immunity of the cervicovagina (Zegels et al., 2009). Another recent study on cervical mucous proteome indicated that plasma proteins were abundant in cervical mucous (Panicker et al., 2010). The majority of proteins identified were categorized under metabolism and immune-response functional groups.

In contrast to plasma samples, some authors discuss the advantages of cervical mucous as a potential source of concentrated biomarkers due to lesser dilution of the sample in CVF-vaginal washings (volume ± 50 mL) in comparison to plasma volume (± 3 L) (Good et al., 2007). In addition, altered biomarker expression patterns in plasma are often not very specific, as they may be associated with different pathologies, because plasma comes in contact with all organs of the body. In contrast, when using CVF samples, it is expected that expression patterns will directly correlate with gynaecological pathologies (Zegels et al., 2009). However, it should be taken into account that CVF is a body fluid that can be highly influenced by many biological factors including menstruation, age, infection, sexual intercourse, usage of contraceptives, pregnancy, etc. Moreover, conditions of CVF collection and the experimental proteomic strategy could affect the proteome results. Although there are promising results, studies in biomarkers in body fluids have not reached consensus on peptides or proteins that have this feature.

3.1.2 Membrane proteomic studies

The main potential of proteomics in cancer research is not only derived from individual experiments, but from comparative studies between different types and states of cancer. There is a need to integrate proteomics, genomics and metabolomics with the aim of achieving a functional and comprehensive interpretation of clinical and pathological data. Comparative proteomic studies have provided tools for establishing some molecular mechanisms of cancer progression, either from normal to neoplastic cells, or from cancer models with different malignant phenotypes. Information obtained through proteomic analysis allows building extensive and comprehensive databases leading to discovery of tumor biomarkers.

Metastasis represents the main cause of death in cancer patients; this multi-step process involves the acquisition of migrating and invasive phenotypes, where the plasma membrane displays a fundamental role. Membrane proteomic studies constitute an analytical challenge due to its heterogeneous composition, dynamic physicochemical characteristics and hydrophobicity; however, 60% of therapeutic targets are directed against membrane proteins. The study of well established cancer cell lines with different phenotypes allows the correlation between the IGF axis expression/activation with membrane protein expression measurements. In particular, cellular motility depends on the adaptive response to environment that extensively relies on key molecular elements of the plasma membrane and cytoskeleton, and therefore usually considered as tumour targets.

Here we present novel results of studies on membrane proteomes of cervical cancer cell lines, differing in viral status and invasive phenotypes: HeLa, an invasive HPV positive cell

line, and C33-A, a non-invasive HPV negative cell line (Garay et al., 2010). C33-A cells express almost exclusively IR-A while HeLa express IGF-IR, IR-A and IR-B. Functional assays showed that IGF-I and IGF-II stimulate migration and invasion in HeLa but not in C33-A cervical cancer cells. In order to make comparisons between proteomic maps, we obtained proteomic maps of the two cell lines by two-dimensional electrophoresis (2-DE), and used the PDQuest (Bio-Rad®) and Progenesis SameSpot (Nonlinear Dynamics Ltd) softwares, to perform between-gel spot comparisons (Fig. 3 A,B). A total of 641 spots in C33-A and 493 spots in HeLa proteomes were identified, with 399 spots matching between the two cell lines. This means that the whole proteomic profiles of cervical adenocarcinoma and cervical carcinoma cells share 80% of similarity, suggesting that the differences in occurrence, prognosis, dissemination and recurrence after treatment, may be related to a limited number of proteins and their relative distribution and expression.

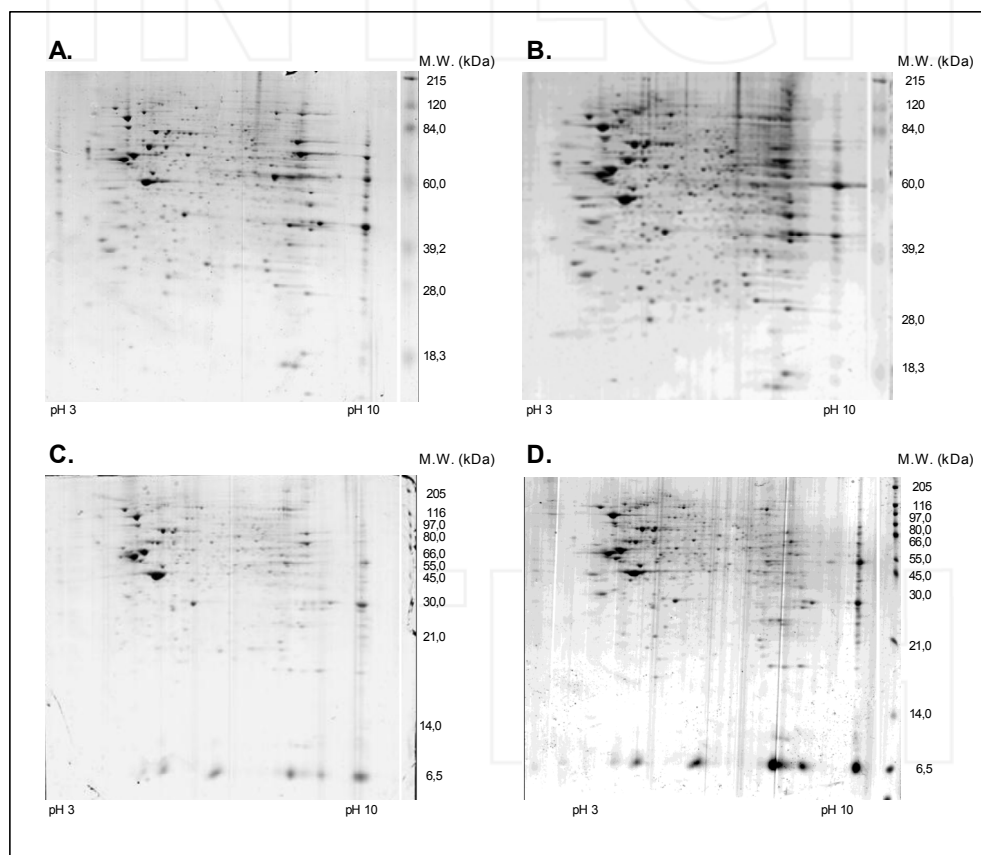


Fig. 3. Proteomes and sub-proteomes of cervical cancer cells. Whole protein extracts from HeLa (A) and C33-A (B) and enriched membrane extracts from HeLa (C) and C33-A (D) were separated by two-dimensional electrophoresis (2-DE) on IPG NL strips pH 3-10 followed by SDS-PAGE and stained with colloidal Coomassie Blue.

Frequently, whole proteomic profiles do not represent the complete map of the expressed proteins in a cell at a given moment; the concentration of some proteins can mask others less abundant but with pivotal biological roles. Study of sub-cellular compartments could improve the identification of a higher number of proteins and consequently a more efficient detection of therapeutic targets. We obtained enriched protein membrane extracts from HeLa and C33-A cells by cell surface biotin labelling followed by avidin purification and separation by 2-DE to obtain the corresponding membrane proteomes (Fig. 3 C, D).

We detected 351 spots in HeLa sub-proteome, out of which 207 spots (41%) were present in the whole proteome and 144 spots corresponded to new proteins not visualized in the total proteome. With respect to C33-A, 395 spots were found in the membrane proteome where 170 spots (43%) matched with the total proteome; additionally 255 new spots were detected. Furthermore, comparison of membrane-enriched proteomes, showed a 58% match between the two cell lines with 272 common proteins, out of which, 86 proteins were found to be differentially expressed (>3-fold). The invasive abilities of HeLa in opposition to C33-A cells, may be related to the observed differences in the membrane protein profile.

In order to identify a higher number of proteins and overcome the limitations associated with the separation of membrane proteins by bi-dimensional electrophoresis, we conducted a multidimensional analysis of enriched membrane fractions. Proteins were separated by SDS-PAGE, each lane in the gel was cut and divided in equal fragments and digested with trypsin. The peptides were separated by reversed-phase liquid chromatography (LC) and analyzed in a hybrid quadrupole mass spectrometer-time of flight (Q-TOF) instrument. Bioinformatics analysis was performed using Mascot Distiler® and Proteome Software Scaffold, which allowed the identification of 44 and 56 proteins in HeLa and C33-A fractions, respectively.

Results showed a differential or exclusive protein expression profile that correlated with cell phenotype. We identified 33 proteins exclusively expressed in C33-A cells that corresponded to cell cycle regulation, metabolism, stress response and immune system evasion, more related to a proliferative non-invasive phenotype. Meanwhile, 22 proteins exclusively expressed in HeLa cells, where mainly involved in cytoskeleton remodelling and associated with cell motility, according to the invasive phenotype. Among the new identified proteins, the more relevant in the regulation of metastatic properties, include: Filamin A and B, Myoferlin and the CD44 - Moesin complex, which have been previously associated with cell adhesion and tumour progression (Feng & Walsh, 2004; Ravid et al., 2008; Bernatchez et al., 2007).

To explore the relationships between the identified proteins and the IGF system, we used the STRING 8.3 database to examine the known or predicted protein-protein interactions among them. The interaction network was built including all the previously identified proteins in the membrane proteome and the members of the IGF system. The interaction network obtained for HeLa cells is visualized in Fig. 4. This analysis confirmed the interaction of some components of the membrane with members of the IGF family, and suggests a potential role in the promotion of the invasive phenotype display by these cells. In particular, this analysis confirmed a direct interaction between IGFBP-3 and the CD44 - Moesin complex, associated with cell adhesion and tumor progression, meaning a promising target for cervical cancer invasion and metastasis control. In conclusion, our

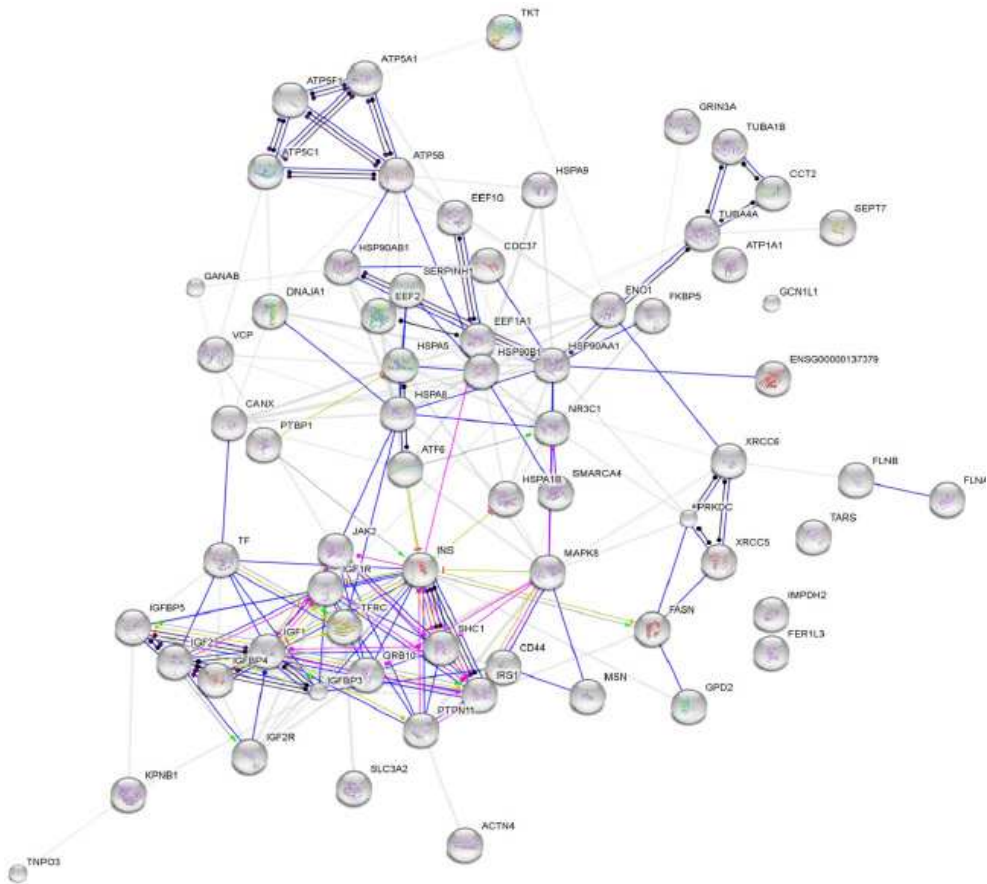


Fig. 4. Protein-protein interaction network representing the associations between the IGF system and identified membrane proteins expressed by the cervical cancer HeLa cell line. The network is visualized by STRING 8.3 database. Each circle represents an individual protein with the recognized abbreviated name. Connecting lines represent association.

approach is unique as it considers the IGF receptors in a wider context within a protein network, and shows that the membrane is a rich reservoir for potential targets for cancer.

4. Conclusion

The evidence reviewed above shows that the IGF system plays a central role in many aspects of the development and progression of cervical cancer. The effects of components of the IGF axis in cervical carcinogenesis share some similarities with those observed in other types of cancer, however, clear differences exist that reflect specificities, possibly due to associations with oncogenic and nononcogenic HPV. The available data support dysregulation of the IGF system expression and signaling, that could promote the progression to a malignant phenotype. It is plausible to consider these molecules as

promising targets for cervical cancer invasion and metastasis control and source of valuable biomarkers of the disease, as deduced from the proteomic approach of membrane targets. Advances in molecular biology and high throughput technologies will improve our understanding of the disease and the search for reliable biomarkers that will facilitate screening, early detection, management, and individualized targeted therapy.

5. Acknowledgment

This work was supported by the Research Council of the Universidad Nacional de Colombia (Grants No. 9173 and 9478) and Colciencias, Colombia (Grant 110145221052). We thank Dr. Patricia Cuervo, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil and Dr. Gilberto B. Domont, Universidade Federal de Rio de Janeiro, Brazil, for the helpful support with the proteomic studies.

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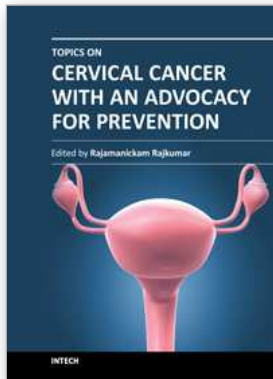
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Topics on Cervical Cancer With an Advocacy for Prevention

Edited by Dr. R. Rajamanickam

ISBN 978-953-51-0183-3

Hard cover, 284 pages

Publisher InTech

Published online 02, March, 2012

Published in print edition March, 2012

Cervical Cancer is one of the leading cancers among women, especially in developing countries. Prevention and control are the most important public health strategies. Empowerment of women, education, "earlier" screening by affordable technologies like visual inspection, and treatment of precancers by cryotherapy/ LEEP are the most promising interventions to reduce the burden of cervical cancer. Dr Rajamanickam Rajkumar had the privilege of establishing a rural population based cancer registry in South India in 1996, as well as planning and implementing a large scale screening program for cervical cancer in 2000. The program was able to show a reduction in the incidence rate of cervical cancer by 25%, and reduction in mortality rate by 35%. This was the greatest inspiration for him to work on cervical cancer prevention, and he edited this book to inspire others to initiate such programs in developing countries. InTech - Open Access Publisher plays a major role in this crusade against cancer, and the authors have contributed to it very well.

How to reference

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Martha-Lucía Serrano, Adriana Umaña-Pérez, Diana J. Garay-Baquero and Myriam Sánchez-Gómez (2012). New Biomarkers for Cervical Cancer – Perspectives from the IGF System, Topics on Cervical Cancer With an Advocacy for Prevention, Dr. R. Rajamanickam (Ed.), ISBN: 978-953-51-0183-3, InTech, Available from: <http://www.intechopen.com/books/topics-on-cervical-cancer-with-an-advocacy-for-prevention/new-biomarkers-for-cervical-cancer-perspectives-from-the-igf-system>

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