



Review

Involvement of CXCL12 Pathway in HPV-related Diseases

Nádia C. M. Okuyama, Fernando Cezar dos Santos, Kleber Paiva Trugilo, and Karen Brajão de Oliveira *

Laboratory of Molecular Genetics and Immunology, Department of Pathological Sciences, Londrina State University, Londrina, PR, Brazil

* **Correspondence:** Email: karen.brajao@gmail.com; Tel: +55-43-337-15728

Abstract: Human Papillomavirus (HPV) is a necessary cause of cervical cancer in women worldwide. However, the HPV infection is not sufficient to cause neoplasia, and immune mediators, such as chemokines, are important in this context, since they are involved in the regulation of leukocyte trafficking in many essential biological processes, including inflammation. Prolonged inflammation is thought to facilitate carcinogenesis by providing a microenvironment that is ideal for tumor cell development and growth. Chemokines also contribute to tumor development by promoting angiogenesis and metastasis. Among these molecules we highlight the chemokine CXCL12, also called stromal-derived factor 1 alpha (SDF1- α), a pleiotropic chemokine capable of eliciting multiple signal transduction cascades and functions, via interaction with either CXCR4 or CXCR7, which have been implicated in malignant cell survival, proliferation and migration. This review will focus on our current knowledge in the pathogenesis of HPV infection, the main aspects of CXCL12 signaling, its participation in tumor development and immunodeficiencies that may enable the HPV infection. We also discuss how *CXCL12* gene expression and polymorphisms may influence tumor development, especially cervical cancer. Finally, we highlight how the inhibition of CXCL12 pathway may be an attractive alternative for cancer therapeutics.

Keywords: CXCL12; polymorphism; HPV; cervical cancer

Abbreviations

3' UTR 3'	Untranslated Region
ACKR3	Atypical Chemokine Receptor 3
AIDS	Acquired immunodeficiency syndrome
AIP4	Atrophin interacting protein 4
AKT	Protein kinase B
APC	Antigen presenting cell
ASCUS	Atypical Squamous Cells of Undetermined Significance
Bcl-2	B cell lymphoma 2
bFGF	Basic fibroblast growth factor
BMDC	Bone marrow-derived cells
CAF	Lymphocytes, cancer-associated fibroblasts
cAMP	Cyclic adenosine monophosphate
CCL2	Chemokine ligand (family CC) 2 C
CCL5	Chemokine ligand (family CC) 5
CCL7	Chemokine ligand (family CC) 7
CCL8	Chemokine ligand (family CC) 8
CCL20	Chemokine ligand (Family) 20
CCR2	Chemokine receptor (family CC) 2
CCR6	Chemokine receptor (family CC) 6
CIN	Cervical intraepithelial neoplasia
CSCC	Cervical squamous cell carcinoma
CXCL5	Chemokine ligand (family CXC) 5
CXCL8	Chemokine ligand (family CXC) 8
CXCL11	Chemokine ligand (family CXC) 11
CXCL12	Chemokine ligand (family CXC) 12
CXCR4	Chemokine receptor (family CXC) 4
CXCR7	Chemokine receptor (family CXC) 7
DAG	Diacylglycerol
DNA	Desoxyribonucleic acid
E	Early
E2F	E2 promoter-binding factor
E6AP	E6-associated protein
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERK1/2	Extracellular signal-regulated kinases 1/2
GAG	Glycosaminoglycan
GATA 2	GATA binding protein 2
G-CSF	Colony-stimulating factor
GDP	Guanosine diphosphate
GEF	Exchange factor of guanine nucleotide

GIRK	G-protein-coupled inwardly rectifying potassium
GTP	Guanosine triphosphate
GPCR	G protein coupled receptor
HECT	Homologous to E6AP carboxy terminus
HIV	Human Immunodeficiency Virus
HSIL	High-grade intraepithelial lesion
HPV	Human Papillomavirus
HR	High risk
HSC	Hematopoietic stem cells
ICL	Idiopathic CD4 lymphopenia
IL17	Interleukin-17
IP3	Inositol 3-trisphosphate
I-TAC	Interferon-inducible T-cell chemoattractant
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
L	Late
LCR	Long control region
LSIL	Low-grade squamous intraepithelial lesion
LTCD4	Lymphocyte T CD4
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage Colony-Stimulating Factor
MMP2	Matrix metalloproteinase 2
mRNA	Messenger RNA
NK	Natural Killer
NF- κ B	Nuclear Factor Kappa B
NSCLC	Non-small cell lung cancer
MMP2	Matrix metalloproteinase 2
MSC	Mesenchymal stem cells
ORF	Open reading frames
P16INK4a	Cyclin-dependent kinase inhibitor
p53	Tumor suppressor protein 53
PBSF	Pre-B-cell growth stimulating factor
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMN	Polymorphonuclear leukocytes
pRB	Retinoblastoma protein
preDC-1	Dendritic cell precursor-1
preDC-2	Dendritic cell precursor-2
PTX	Pertussis toxin
SDF-1	Stromal cell-derived factor 1
SNP	Single nucleotide polymorphism

STAT	Signal transducer and activator of transcription
TAM	Tumor-associated macrophages
TAN	Tumor-associated neutrophils
TCR	T cell receptor
USP14	Ubiquitin-specific protease 14
VEGF	Vascular endothelial growth factor
WHIM	Warts, hypogammaglobulinemia, infections, myelokathexis
ZT	Transformation zone

1. Introduction

Cervical carcinoma is considered an important public health issue. It is the third most common type of cancer in Brazilian women [1] and the fourth in women worldwide [2]. The disease is strongly associated to Human Papillomavirus (HPV) infection, which is present in 99.7% of the cancer cases [3]. There are more than 200 types of the virus and they are classified according to their carcinogenic potential as high-risk, undetermined risk and low-risk [4]. The majority of invasive cervical cancer is associated with HPV16 and 18 [5], both types discovered by Harald Zur Hausen in the 1980s. HPV is also responsible for other types of cancers, such as vulva [6], vagina [7], anus [8], and oropharynx [9], as well as benign diseases such as genital warts and recurrent respiratory papillomatosis [10].

2. HPV Infection

Human Papillomavirus (HPV) is a small non-enveloped deoxyribonucleic acid (DNA) virus that belongs to *Papillomaviridae* family. The doubled-stranded DNA has around 8000bp and consists of eight open reading frames (ORF's) and one non-coding region named long control region (LCR). The ORF's contain seven genes called Early genes (E) and two Late genes (L). Early genes are responsible for the viral replication and late genes for encoding the proteic capsid (Figure 1-A) [11].

The virus may be in episomal state or integrated in the cell genome. In episomal state, high levels of HPV E2 protein suppress the E6 and E7 protein expression. Viral DNA integration usually disrupts E2 expression, leading to the deregulated expression of early viral genes, including E6 and E7, as well as increased proliferative capacity, a crucial step in progression to cancer (Figure 1-B) [12].

E4 protein participation is not fully understood and it is expressed primarily during the late stages of infection, at or around the time that genome amplification begins. It appears to be involved in new virus release and transmission [13]. Many types of HPV show a conserved E5 gene. It appears to play a significant role in virus cell cycle life and is still coevolved with the major HPV oncogenes E6 and E7 [13]. Both proteins, E6 and E7, are strongly expressed in HPV-carrying anogenital malignant tumors [11,14]. E7 protein binds to retinoblastoma protein (pRb), which is a cell cycle down-regulator. Once at its hypophosphorylated state, pRb releases the E2

promoter-binding factor (E2F) at the nucleus, enabling the entry into the S phase of the cell cycle. E7 involvement in the cell cycle is reinforced by E6 protein activity. E6 interacts with E6AP, a cellular ubiquitin ligase, and directs the ubiquitination activity of E6AP especially toward tumor suppressor protein 53 (p53), an important molecule for apoptotic control, driving it to degradation by proteasome [15]. Furthermore, E6AP can also function as a ligand-activated coactivator for the estrogen receptor (ER). The phosphorylated ER binds to E6AP and then, the formed complex is recruited to activate the transcription of a subset of ER target gene promoters [16]. There is strong evidence that estrogen contributes to cervical carcinogenesis through its nuclear ER α . This hormone-receptor axis is required for cervical cancer genesis and persistence, since estrogen seems to be able to increase the expression of the E6 and E7 HPV oncogenes [17,18]. In this sense, the participation of E6AP as ER coactivator and in p53 proteolysis, suggests that the estrogen signaling pathway could be crosstalking with HPV carcinogenesis mechanisms mediated by E6AP in the development of cervical cancer.

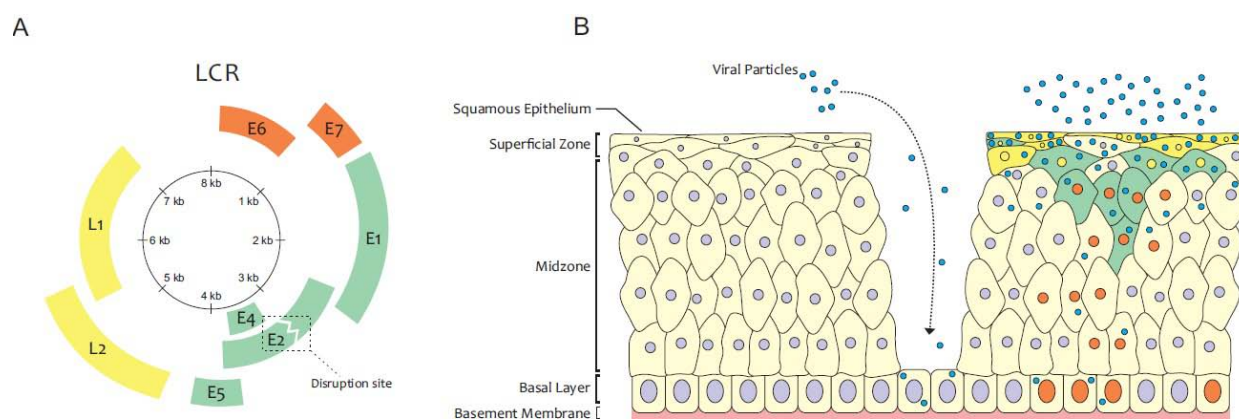


Figure 1. The human papillomavirus genome and the virus life cycle. A) HPV is a double-stranded circular DNA virus. Its genome (about 8000 bp) has 6 early ORFs (E1, E2, E4 and E5 [green]) and E6 and E7 (orange), so called because they are expressed mostly in the early stages of infected keratinocytes differentiation. The late genes L1 and L2 (yellow) code the viral capsid proteins, produced late in the infection. The Long Control Region (LCR) has regulatory elements and transcription factor binding sites that are important in determining and controlling the viral gene expression. The figure depicts the region where viral DNA disruption occurs when HPV integrates into the host genome, enabling cell transformation by overexpression of E6 and E7 oncoproteins. **B)** The HPV is an epitheliotropic and mucosotropic virus that infects basal cells that were exposed by a microwound. Early genes are expressed and viral DNA is productively replicated from episomal DNA. In the upper layers, the late genes are expressed and viral particles are assembled and released. Notice that the viral genes in figure B in each phase of expression are colored according to the genes shown in Figure A.

HPV is transmitted mainly by skin-to-skin or mucosa-to-mucosa contact. Different types of HPV may be transmitted at the same time due to their common route of transmission [17]. Risk factors such as age of the first intercourse, number of sexual partners, condom use, long-term oral contraceptive use, multiparity and smoking are all important for the development of HPV related lesions, but the reason why they function as cofactors is not well established [18].

Most women are infected by at least one type of HPV during their sexual life. The infection may be asymptomatic and may be suppressed by the immunological system in 18 months [18]. Infection occurs through micro wounds in the basal layer and the virus infects cells of the epithelium via a non-established receptor. Infected basal cells migrate to the upper layers of the epithelium and start to differentiate. Then, the virus DNA is packaged forming new capsids and virions are released from the cell [19]. Persistent HPV infection occurs only in a minority of women and evolves to low-grade squamous intraepithelial lesion (LSIL) or high-grade intraepithelial lesion (HSIL) which can still regress or progress to invasive cervical carcinoma [20].

Therefore, infection with high-risk HPV is necessary but not sufficient for cell immortalization and subsequent malignancy. Genetic modifications in the host due to viral DNA integration and chemical and physical mutagens may also contribute to these processes [17]. In addition, exogenous and endogenous factors including tobacco use, parity, oral contraceptive use [20], immune system impairment, and immunological interactions at the site of infection [21] may all influence progression from HPV infection to high-grade cervical lesions.

In this context we highlight the chemokines, since evidence suggests that chemokines are important regulators in the development of viral infections [22] and are also responsible for inducing directional cellular migration, particularly of leukocytes during inflammation, since this prolonged inflammation is thought to facilitate carcinogenesis by providing a microenvironment that is ideal for tumor cell development and growth [23].

3. Chemokines and Cancer Microenvironment

The term chemokines, a short form of “chemotactic cytokines”, was coined in 1992 at a gathering in the elegant castle of Baden near Vienna, after the International Immunology Meeting in Budapest [24]. Chemokines are small proteins from the immune system, which have chemotactic activity that stimulates the migration of different cell types such as lymphocytes, monocytes, neutrophils, endothelial cells, mesenchymal stem cells, and malignant epithelial cells. They constitute the largest family of cytokines, consisting of approximately 50 endogenous chemokine ligands in humans and mice. Chemokines are divided into four subfamilies based on the position of the first two N-terminal cysteine residues, including the CC, CXC, CX3C, and XC subfamilies [24]. Chemokine receptors constitute the largest branch of the γ subfamily of rhodopsin-like seven-transmembrane receptors. Chemokine receptors are differentially expressed on all leukocytes and can be divided into two groups: G protein-coupled chemokine receptors (GPCR), which signal

by activating pertussis toxin (PTX)-sensitive Gi-type G proteins, and atypical chemokine receptors, which appear to shape chemokine gradients and dampen inflammation by scavenging chemokines in a G protein-independent manner. There are approximately 20 signaling chemokine receptors and 5 non-signaling chemokine receptors [24,25].

Normal tissues carefully control the production and release of growth-promoting signals that instruct entry into and progression through the cell growth and-division cycle, thereby ensuring a homeostasis of cell number and thus maintenance of normal tissue architecture and function. Cancer cells, by deregulating these signals, become masters of their own destinies [27]. Several studies have shown that chemokines and their receptors are implicated in tumor growth and progression. For example, genetic silencing or pharmacologic inhibition of CXCR7 reduced breast tumor growth in mice. Furthermore, MAPK/ERK signaling pathways are downstream targets of the CXCL12/CXCR7 pathway [28].

Another chemokine-mediated mechanism that contributes to tumorigenesis is angiogenesis, a discrete step that is required to allow tumor propagation and progression and the induction of a tumor vasculature [29]. Like normal tissues, tumors require an adequate supply of oxygen, metabolites and an effective way to remove waste products. These requirements vary, however, among tumor types, and change over the course of tumor progression. But gaining access to the host vascular system and the generation of a tumor blood supply are rate-limiting steps in tumor progression [29]. In this way, chemokines and their receptors have been demonstrated as mediators of the vasculogenic process. CXCR4 is expressed in developing vascular endothelial cells and mice lacking CXCR4 or CXCL12 have defective formation of the large vessels that supply the gastrointestinal tract [30], supporting the idea that CXCL12 is a crucial chemokine for the development of new blood vessels. Investigations have demonstrated that low constitutive levels of CXCR4 expression by endothelial cells can be up-regulated by at least 4-fold by the vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), rendering endothelial cells more responsive to CXCL12 [31,32].

Tumor progression towards metastasis is often depicted as a multistage process in which malignant cells spread from the original tumor to colonize distant organs. The classical simplification of metastasis into an orderly sequence of basic—local invasion, intravasation, survival in the circulation, extravasation and colonization steps—has helped to rationalize the complex set of biological properties that must be acquired for a particular malignancy to progress towards overt metastatic disease [33]. Not only can tumor cells travel around the body, but they do so under the influence of signals that determine their migratory behavior. A successful metastasis at a new destination requires two stages: first, the cells must respond to chemotactic signals that lead them to “hospitable ground”; and second, they must survive and thrive upon arrival [34]. Chemokines are likely to participate in both processes. Initial evidence that pointed to a role for chemokines in metastasis came from the observation that the expression of chemokine receptors by tumor cells is not random; that is, tumor cells only express selected chemokine receptors [34]. The CXCL12/CXCR4 axis is strongly involved in metastasis. In vivo, neutralizing the interactions of

CXCL12/CXCR4 significantly impairs metastasis of breast cancer cells to regional lymph nodes and the lungs [35]. Malignant melanoma, which has a similar metastatic pattern to breast cancer but also a high incidence of skin metastases, shows high expression levels of CCR10 in addition to CXCR4 and CCR7 [35]. Using CXCR4 antagonists or CXCL12-specific blocking antibodies, many studies have shown these observations in a variety of cancer models, including models of breast cancer, prostate cancer, lung cancer, colorectal cancer, gastric cancer, and glioblastoma [35]. In glioblastoma, CXCL12 has been reported to have direct growth effects mediated through CXCR4. The involvement of CXCR4 in glioblastoma is another example of tumor cells hijacking physiological processes because the CXCL12-CXCR4 axis is important for CNS development and both CXCL12 and CXCR4 are highly expressed in the CNS [36].

Chemokines are emerging as key mediators not only in tumor growth enhancement, blood vessel formation and metastasis, but also in the recruitment of a number of different cell types to the tumor microenvironment. This includes infiltrating cells such as tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs) and lymphocytes, cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSCs) and endothelial cells [37]. In ovarian cancer, for example, high levels of CXCL12 produced by tumor cells can de-regulate immunity by attracting dendritic cell precursor-2 (preDC2), which does not appear to mediate effective anti-tumor activity, and by altering preDC1 type distribution, immunity and fibrosis stimulation. The result is lack of dendritic cell maturation and antigen presentation failure [37,38].

4. CXCL12 Signaling Pathways

CXCL12 is an important α -chemokine that binds to the G-protein-coupled seven-transmembrane receptor CXCR4. For many years, it was believed that CXCR4 was the only receptor for CXCL12. However, several reports recently provided evidence that CXCL12 also binds to another seven-transmembrane receptor called CXCR7, sharing this receptor with another chemokine family, CXCL11 [39].

The CXCL12-CXCR4 axis may activate many downstream signaling cascades beginning through heterotrimeric G proteins as well as β -arrestins (Figure 2) [40].

Once activated, G protein inhibits adenylyl cyclases and cAMP production and stimulates the activity of the Src family tyrosine kinases that activate the Ras/Raf/MEK/ERK pathway, modulating cell cycle progression through the phosphorylation of the adaptor protein Shc. In parallel, CXCR4-oriented migration is mediated by the phosphatidylinositide 3-kinases (PI3Ks). PI3Ks regulate gene transcription, cell migration and cell adhesion by phosphorylating AKT and several focal adhesion components. Furthermore, phospholipase C (PLC) is activated which, in turn, catalyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) hydrolysis into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ production results in Ca²⁺ mobilization from the intracellular stores, while DAG promotes the activation of protein kinase C (PKC) and mitogen associated protein

kinase (MAPK) [26]. CXCL12/CXCR4 also can activate cell proliferation via modulating the Wnt canonical pathway, and suppress apoptosis by NF- κ B activation [41,42].

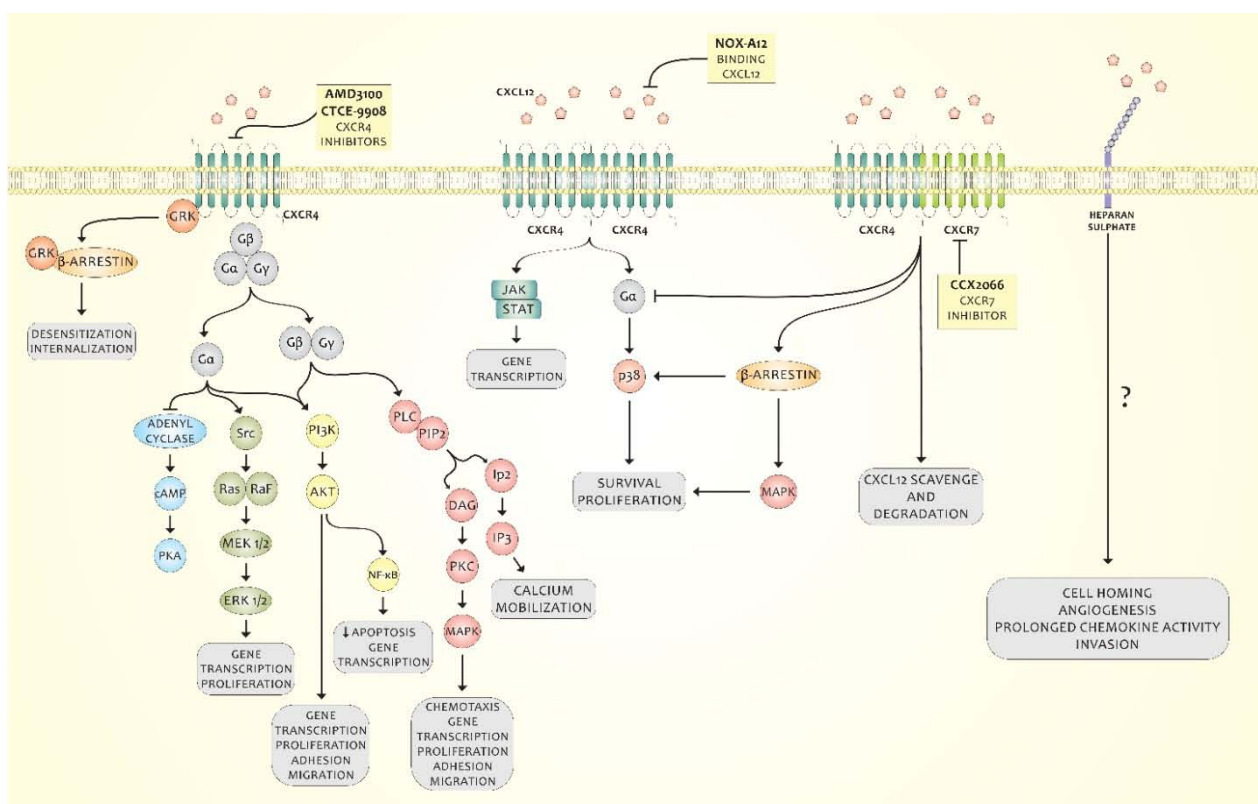


Figure 2. CXCL12/CXCR4/CXCR7 Signaling Pathway. CXCL12 binding to CXCR4, which may form homodimer, triggers G-protein-coupled signaling and subsequent activation of the PI3K/AKT, PLC/IP3, ERK1/2 pathways, resulting in gene transcription, cell adhesion, migration, proliferation, and cell survival. The β -arrestin pathway can be activated through GRK, required for CXCR4 internalization. CXCR4 oligomerization can also activate a G-protein independent pathway via JAK/STAT, inducing gene transcription; p38 may also be activated modulating survival and proliferation. CXCL12 binding to CXCR4-CXCR7 heterodimers can inhibit $G\alpha$ signaling and potentiates the β -arrestin-dependent downstream signaling, activating p38 and MAPK to increase cell survival. In the latter case, CXCR7 changes the conformation of the CXCR4/G-protein complexes and abrogates signaling. In addition, activation of the β -arrestin pathway may lead to scavenging and degradation of CXCL12. Binding of CXCL12 to heparan sulfate present on cell surface and extracellular matrix prevents its proteolysis and mediates events such as migration, angiogenesis and cancer invasion. The figure depicts drugs tested in clinical and preclinical studies capable of blocking the CXCL12 pathway through receptor inhibition or ligand binding.

CXCR4 activation and phosphorylation can also lead to a dynamic ubiquitination/deubiquitination cycle. According to Bhandari et al. [43], the ubiquitination mechanism is mediated through recruitment of the E3 ubiquitin ligase atrophin interacting protein 4

(AIP4), after CXCR4 activation. A member of the Nedd4-like homologous to E6AP carboxy terminus (HECT) domain family of E3 ubiquitin ligases, AIP4 interacts directly with the C-tail of CXCR4 and ubiquitinates nearby lysine residues, enabling the receptor to be targeted for lysosomal degradation and resulting in CXCR4 down-regulation. While ligand-dependent CXCR4 ubiquitination accelerates CXCR4 degradation, mechanisms for receptor deubiquitination are also activated. CXCL12-CXCR4 binding induces a time-dependent association of CXCR4, or at least its C terminus domain, with ubiquitin-specific protease 14 (USP14), a member of the deubiquitination catalytic family, which deubiquitinates this receptor [44]. These mechanisms seem to be important factors in the regulation of CXCR4 membrane expression and consequently in CXCL12 signaling.

The process of homologous desensitization, or becoming refractory to continued stimulation, is initiated by G protein-coupled receptor kinase (GRK) phosphorylation of serine/threonine residues of the third intracellular loop (TIL) or cytoplasmic tail (C-tail) following receptor activation. This phosphorylation allows for the subsequent binding of arrestin-2 and/or arrestin-3, effectively uncoupling the receptor from further G protein activation and often targeting the receptor for internalization [45].

Moreover, CXCR4 can trigger a G-protein independent signal pathway through association with β -arrestins [46]. It has been reported that arrestin-2 and -3 enhance CXCR4-mediated ERK activation and arrestin-3 is involved in p38 activation and migration following CXCL12 stimulation, thus promoting cell migration [45]. CXCR4 oligomerization has been postulated to play a role in modulating GPCR signaling. Both CXCR4 homodimers and heterodimers have been reported. Homodimerization of CXCR4 has been suggested to result in G-protein-independent signaling through the JAK/Stat signaling pathway [47,48].

In addition to binding to CXCR4, CXCL12 binds to the non-canonical CXCR7 receptor, also known as ACKR3. Depending on the context, CXCL12-CXCR7 binding is thought to result in two different responses: ligand internalization followed by ligand degradation (chemokine clearance), and β -arrestin-mediated G protein-independent signaling. The chemokine clearance function is supported since CXCR7 does not associate with heterotrimeric G proteins, and ligand binding does not evoke calcium influx. The GPCR amino acid sequence motif, DRYLAIV, which is located in the second intracellular loop and is crucial for $G_{\alpha i}$ protein coupling and calcium signaling, is notably lacking in CXCR7. Instead, CXCR7 harbors a DRYLSIT motif, which strongly suggests that this structural difference contributes to CXCR7's inability to relay intracellular signals through $G_{\alpha i}$ [49]. In addition, CXCR7 binds CXCL12 with an affinity ten times higher than CXCR4, and the receptor is rapidly recycled back to the plasma membrane upon ligand-induced internalization. These observations suggest that CXCR7 can act as an efficient chemokine sink because it can successfully compete with CXCR4 for access to the ligand. At least two processes involving CXCR7 may modulate CXCL12 bioavailability. CXCR7 can scavenge CXCL12 and mediate ligand-induced internalization and degradation, thus removing it from the extracellular environment, or modulate the balance between monomeric and dimeric forms of CXCL12 [50]. The co-expression of CXCR7 with

CXCR4 in the same cell may result in the sequestration of CXCL12 from the microenvironment rather than its binding to CXCR4. Mechanisms that alter the overall availability, distribution, and gradients of CXCL12 in tumor microenvironments should, in principle, regulate the functions of CXCR4 in tumor growth and metastasis [50].

Data suggest that CXCL12 may be secreted in two forms: monomer or dimer. The two forms may have distinct roles but their respective actions have not yet been fully elucidated. The balance between the two forms is controlled by factors such as basic pH, the presence of multivalent anions and high concentration of glycosaminoglycans (GAGs) at the cell surface which favor formation of the dimeric form [51]. CXCR7 is also a factor that controls the balance of CXCL12 monomers and dimers because of its preference to link to the CXCL12 monomer [52].

CXCR7 has a role in modulating CXCR4 expression and function. Whether CXCR7 activates or impairs CXCR4 effects depends on the final outcome of their crosstalk. Although GPCRs were previously assumed to act as monomers, recent structural and computational modeling studies have revealed that GPCRs such as CXCR4 can form homodimers and heterodimers, either constitutively or upon ligand binding. GPCR dimer structures have been extensively studied through crystal structure analysis, which can be used to predict the dimerization of the receptor that determines its biological activity and pharmacological effects *in vivo* [50,53].

Putative CXCR4/CXCR7 heterodimers have been proposed to be important regulators of ligand-dependent signaling [54].

The formation of functional heterodimers has been demonstrated using bioluminescence resonance energy transfer assays. Dimerization may result in the recruitment of b-arrestin to the CXCR4/CXCR7 complex [50]. However, CXCR7 may also activate G-protein-independent signaling pathways, as β -arrestin pathway, eliciting pERK signaling to modulate cell fate and migration, what was demonstrated in several systems [50].

Taken together these findings suggest atypical signal properties in CXCR7 leading to cell growth and survival in the presence of CXCL12. The molecular mechanisms which govern the responses are unclear and distinct from common chemokine receptor-mediated signaling [55].

Thus, with CXCR7 identified as a new receptor for CXCL12, the role of the CXCL12-CXCR4 axis in regulating several biological processes became more complex.

Another level of complexity is added by the fact that chemokine function, transport, clearance and degradation could be affected through chemokine binding to GAG, which is present on all animal cell surfaces and in the extracellular matrix [56]. GAGs, in particular heparan sulfate (HS), play a fundamental role in forming the chemokine concentration gradients to establish directional signals for migrating cells. Electrostatic contacts between HS and chemokines determine both the affinity and the specificity of the molecular interactions that are supposed to modulate the *in vivo* biological activity of chemokines complexed to this proteoglycan. It has been shown that the high affinity association of HS with CXCL12, besides locally concentrating the chemokine, prevents its proteolysis [57,58], thus contributing to the increased stability and prolonged immobilization and

activity of CXCL12 in tissues, which can enhance directional migration and tissue homing of cells [57]. Thereby, binding with GAGs seems to regulate important events in both homeostatic and pathologic conditions, such as angiogenesis [57] and cancer invasion [59].

5. CXCL12 Gene and Cancer

The *CXCL12* gene is located on chromosome 10q.11.1 which was mapped by fluorescence *in situ* hybridization that differs from the location of loci of other known chemokine family members' genes that had been distinguished by chromosomal localizations: genes encoding members of the CC and CXC subfamilies are located on chromosome 17q and 4q, respectively [60]. It was first cloned from a bone marrow-derived stromal cell line and was later identified as a pre-B-cell growth stimulating factor (PBSF). The *CXCL12* gene may have a single nucleotide polymorphism (SNP) in the 3'UTR (3'-untranslated region) also known as rs1801157 (*CXCL12* 801 G → A) described for the first time in 1998 by Cheryl Winkler [61] who studied patients with AIDS. *CXCL12* polymorphism rs1801157 is also associated to coronary heart disease [62], a possible late-stage protective effect on HIV-1 disease progression in the Brazilian population [63], and as mentioned before, elevated risk of many types of cancer development including breast, lung and lymphoma [64]. Significant frequency of rs1801157 *CXCL12* polymorphism AA genotype was observed in patients with renal cell carcinoma and the overall survival was shorter compared to patients presenting GG and GA genotypes [65]. According to Shimanski et al. [66], patients presenting AA and GA genotypes are more susceptible to distant metastasis development in esophagogastric cancer.

The biological role of *CXCL12* polymorphism in cancer prognosis is controversial. According to Razmkhah et al. [67], this polymorphism is associated with an increased susceptibility to breast cancer development. Combination of low levels of the chemokine and its polymorphism may identify patients with an intrinsic susceptibility to poor survival [68]. In line with this, *CXCL12* level measurement may be an important biomarker to cancer prognosis. Although *CXCL12* mRNA has been reported to be expressed in many different tissues, quantitative analysis of *CXCL12* expression in many human organs of patients with breast cancer revealed that *CXCL12* mRNA is expressed preferentially in lymph nodes, the lungs, liver and bone marrow. Phillips et al. [69], using an animal model of xenograftment of human non-small cell lung cancer (NSCLC) cells, found that *CXCL12* protein levels are significantly higher in the adrenal glands, the lungs, liver and bone marrow, that are known to be highly susceptible to human NSCLC metastasis. Moreover, the plasmatic level of *CXCL12* was increased in patients with prostate cancer compared to those who had a benign form of the disease and health controls [70] and in tumoral bone marrow cells in multiple myeloma [71].

Furthermore, Sei et al. [72] demonstrated that *CXCL12* mRNA may be up-regulated due to the gene variation located within 3' UTR suggesting that *CXCL12* polymorphism may influence the abundance of the chemokine. However, de Oliveira et al. [73] observed that allele A breast cancer

patients presented a mRNA CXCL12 expression about 2.1-fold smaller than GG breast cancer patients, suggesting that allele A is associated with low expression of CXCL12 in the peripheral blood from ER-positive breast cancer patients.

Little is known about the chemokine CXCL12 and its polymorphisms in cervical cancer and the published data so far are controversial.

6. Involvement of CXCL12 Axis in HPV Infection Susceptibility

Host defense against HPV relies on intact and functioning cellular immunity, including T-cell and natural killer (NK) cell cytotoxicity. Patients in whom warts are severe or recalcitrant, concern for immune defects is raised [74].

The WHIM syndrome features susceptibility to human Papillomavirus infection-induced warts and carcinomas, hypogammaglobulinemia, recurrent bacterial infections, B and T-cell lymphopenia, and neutropenia associated with retention of senescent neutrophils in the bone marrow (i.e. myelokathexis). WHIM syndrome patients present abnormal expression of CXCL12 and its receptors in keratinocytes immortalized by HPV16 and 18 in a dependent way of E6 and E7 oncoprotein expression. E6 and E7 proteins expressed in the suprabasal layer would positively regulate CXCL12 levels and its receptor, which would raise cell proliferation and viral replication. This phenomenon may occur for low and high risk HPV and could be enhanced by CXCR4 mutation that may provide a base for development of WHIM verrucose patients and HPV associated to oncogenesis [75]. A key marker seen in the WHIM syndrome is the increased activation of the CXCL12/CXCR4 axis, which usually results from gain-of-function mutations in CXCR4. CXCR4 mutants maintain association with β -arrestins and trigger abnormal β -arrestin-dependent pathways as revealed by activation of the ERK1/2 signaling. Although the immuno-hematological manifestations of the WHIM syndrome apparently result from CXCR4 dysfunctions, the mechanisms by which these dysfunctions might affect leukocyte homeostasis remain unknown [76].

GATA2 deficiency is associated with impaired membrane expression and chemotactic dysfunctions of CXCR4. Patients suffering from GATA2 deficiency also display a high susceptibility to HPV infections, as do patients with the WHIM syndrome. CXCR4-dependent chemotactic responses in patients with GATA2 deficiency are differently affected among lymphocytes with a marked loss of function for NK cells but an enhanced function for B lymphocytes. These dysfunctions may contribute to the physiopathology of this deficiency by affecting the normal distribution of lymphocytes and thus potentially affecting the susceptibility of patients to associated infections [77].

In Idiopathic CD4 lymphopenia (ICL) cryptococcosis, HPV, and nontuberculous mycobacterial infection were the three most common infections. The couple CXCL12/CXCR4 directs thymopoiesis and regulates the domiciliation of lymphocyte T (LT) in secondary lymphoid organs. Loss or gain of CXCR4 expression in mice leads to a decrease in LT CD⁴⁺ associated with a defect in thymic

maturation and exacerbates migration to the bone marrow [78]. Warts in patients with ICL are typically presented as disseminated verrucae or flat warts on the extremities, face, and genitals. A few reports have found HPV types 2, 3, 6, and 49 in cutaneous lesions. HPV-related dysplasia and carcinoma can be the presentation of ICL [74]. These results underscore the importance of delineating the mechanisms underlying CXCL12/CXCR4 dysfunctions, including their penetrance and potential role in the pathogenesis of rare deficiencies. Host defense against HPV is multifaceted. Investigation of patients with severe recalcitrant HPV has increased our understanding of host defense and cutaneous and systemic immunity [74,77].

7. CXCL12 and Cervical Cancer

Five polymorphisms of the *CXCL12* gene were analyzed in a based-population case-control study where women with invasive squamous carcinoma and adenocarcinoma *in situ* were investigated. The authors concluded that *CXCL12* polymorphism rs1801157 was not associated with cervical cancer risk [79]. According to Tee et al. [80] the same polymorphism raises no susceptibility to neoplastic lesions of the uterine cervix. No significant difference of genotype frequencies of the *CXCL12* polymorphism was found between patients and controls. However, a stratified analysis between the *CXCL12* rs1801157 genotype distribution and cervical cancer risk showed that the polymorphism may be a risk factor for patients with a positive history of tobacco smoking [81].

A few studies were performed in order to elucidate the transcriptional pattern of the *CXCL12* gene in cervical cancer. Jaafar et al. [82] have shown overexpression of *CXCL12* in squamous and glandular cervical lesions. This increased expression was associated with infiltration of FOXP3⁺ regulatory T cells (known to be associated with cervical cancer development), cancer staging, histopathological progression and HPV infection, indicating that CXCL12 could be a good marker for clinical progression. Also, Huang et al. [83] demonstrated that the expression of CXCL12 and its receptor CXCR4 was significantly higher in the cervical cancer group than in the Cervical intraepithelial neoplasia CIN1 and CIN2 groups. CXCR7 was also higher expressed in cervical cancer than in CIN and normal cervical mucosa, especially in those patients with advanced staging and lymph node metastasis. CXCL12 appeared to be positively regulated by CXCR7 at the post-transcriptional level in CSCC (cervical squamous cell carcinoma) [84]. Zanotta et al. [85] studied the soluble immune mediators in young women with HR-HPV (High Risk HPV). Data showed that CXCL12 was significantly linked with HR-HPV infection and increased levels have been detected in women with pre-cancerous lesion.

Correlation between overexpression of CXCL12/CXCR4 and lymph node metastasis was also observed by Wei et al. [86]. A variable number of tumor cells in 33 specimens expressed CXCL12. The overexpression rate of CXCL12 was significantly higher in IB cases than in IIB cases or in tumors with lymph node metastasis. The exact mechanism of CXCL12 and its receptor CXCR4 in cervical cancer metastasis development still needs to be fully elucidated.

In order to investigate the role of the CXCL12/CXCR4 axis in mediating metastasis in cervical cancer cells, Shen et al. [87] analyzed the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in HeLa cells after binding CXCL12 to CXCR4 and observed that, in HeLa cells exposed to CXCL12, ERK-1/2 was rapidly phosphorylated, that the adhesion ability of HeLa cells to fibronectin and laminin was increased after CXCL12 treatment while pretreatment of HeLa cells with an ERK-1/2 inhibitor, PD98059, decreased adhesion of HeLa cells to the extracellular matrix with the presence of CXCL12. They also observed that increased amounts of active matrix metalloproteinase 2 (MMP2) were secreted in response to increased CXCL12 concentrations, suggesting therefore that CXCL12/CXCR4 participates in tumor invasiveness and metastasis in cervical cancer through regulating the adhesion ability by activating the MAPK signaling transduction pathway and promoting MMP2 secretion. The events discussed above are summarized in Figure 3.

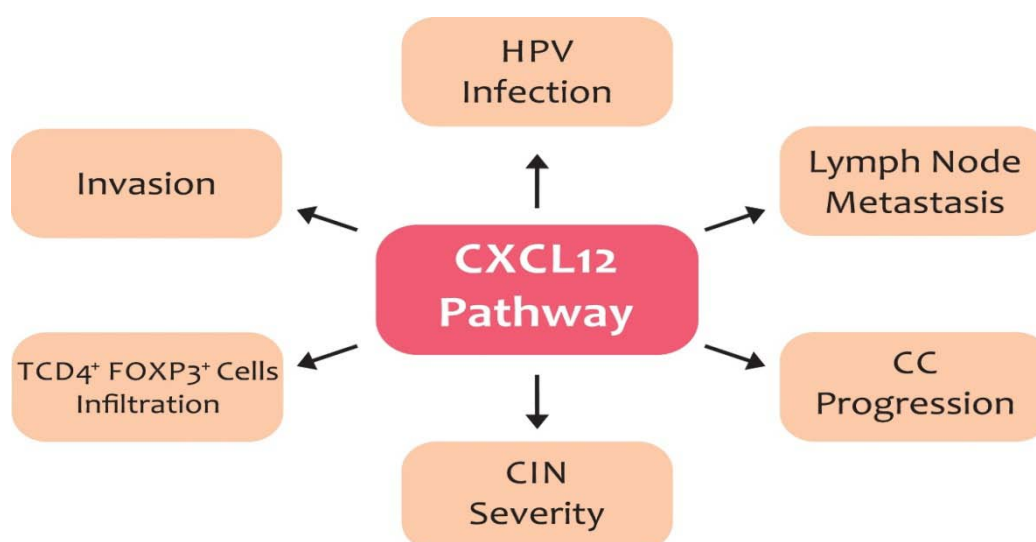


Figure 3. Events mediated by CXCL12 pathway in HPV infection and carcinogenesis.

HPV: human papillomavirus; CC: cervical cancer; CIN: cervical intraepithelial neoplasia.

Yadav et al. [88] investigated whether deregulation in CXCR4 signaling (as a consequence of deregulated CXCL12 expression) modulates the metastatic potential of cervical carcinoma cells, and observed a controversial result suggesting the tumor suppressor functions of CXCL12 in cervical cancer. They demonstrated that CXCL12 is frequently downregulated and its promoter is hypermethylated in cervical cancer cell lines and primary tumor biopsies. This suggests that (a) silencing of CXCL12 in cervical cancer cells may be critical in migration and invasion, the key events in cancer cell metastases; (b) cervical cancer cells having downregulated CXCL12 are more prone to being attracted to CXCL12 expressed at secondary sites of metastases; and (c) CXCL12 inhibits anchorage independent cell growth via anoikis.

Since CXCL12, through its receptors, CXCR4 and CXCR7, plays critical roles in mediating tumor metastasis and angiogenesis in several types of cancers including cervical cancer, the CXCL12/CXCR4/CXCR7 axis is a potential target for therapeutics that block the CXCL12/CXCR4 or CXCL12/CXCR7 interactions or which inhibit activities of downstream signaling.

8. CXCL12/CXCR4/CXCR7 Blockade

Considering the significance of the CXCL12 pathway in cancer development, some drugs were developed to impair this axis and showed promising results. These drugs comprise AMD3100, or plerixafor (Mozobil; Sanofi SA, Paris, France), a CXCR4 antagonist; CTCE-9908 (Chemokine Therapeutics Corp, Vancouver, BC, Canada), a CXCL12 analog; Nox-A12 (Noxxon Pharma AG, Berlin, Germany), an anti-CXCL12 aptamer; and CCX2066 (ChemoCentryx, Inc), a CXCR7-specific inhibitor [89–91]. There are reports using interference RNA, that might be a potential therapy tool [92–94]. Of these, the use has been clinically approved [90,95] of AMD3100 for stem cell mobilization in patients with non-Hodgkin's lymphoma and multiple myeloma and CTCE-9908 for patients with osteosarcoma. AMD3100 is the better studied drug among agents that antagonize CXCR4. Initially researched as an anti-HIV drug, AMD3100 has already been shown to decrease metastatic potential in animal models for different types of tumors, including breast, ovarian, colorectal cancer and melanoma [46]. In an HPV-induced disease context, Uchida et al. [95] observed that treatment with AMD3100 inhibited lymph node metastases of the oral squamous cell carcinoma cell line (B88) when they were orthotopically inoculated into nude mice. It is known that oral squamous cell carcinoma is highly associated with HPV infection [96]. Furthermore, in a transgenic mouse model of HPV-induced epidermal neoplasia, daily treatment with AMD3100 abrogated CXCL12 and cyclin-dependent kinase inhibitor p16INK4a coexpression in dysplastic epidermis [97]. p16INK4a is considered a marker of virus-induced cell cycle deregulation [98]. The same study also reported reduced papilloma development on the ears and reduced ear thickness of the treated mice, chronic inflammation impairment in the course of premalignant progression and decreased proliferation and increased apoptosis of keratinocytes [97]. Administration of AMD3100 also is a clinical indication for the WHIM syndrome [26].

Studies on the blockade of the CXCL12 pathway differ and must be carefully analyzed. Experimental studies have shown that anti-CXCL12 drugs can be effective in decreasing tumor growth and metastasis in human and animal models at the beginning of treatment or tumor establishment, but low efficiency in established tumors [99–101], although tumor growth was minor in brain and prostatic human cancers [102,103]. Otherwise, the CXCL12 blockade might be more useful in metastasis prevention. Dose-dense adjuvant chemotherapy is given to women with breast cancer together with granulocyte colony-stimulating factor (G-CSF), and G-CSF may induce proteolytic disruption of the CXCL12/CXCR4 axis, inhibiting CXCR4-dependent homing micrometastatics [104]. Furthermore, clinical and preclinical studies demonstrated that treatment

with some antiangiogenic (e.g.: cediranib), cytotoxic (e.g.: paclitaxel) and vascular-disrupting agents (e.g.: OXi-4503) drugs may increase circulating CXCL12 and bone marrow-derived cell (BMDC) infiltration, thus favoring angiogenesis and metastasis [105–108]. Moreover, concomitant therapy of anti-CXCR4 AMD3100 and radiotherapy delayed tumor growth and increased tumor clearance in brain, lung and breast cancers in xenograft models [109,110]. Some clinical studies also showed high CXCL12, CXCR4 and CXCR7 expression in advanced glioblastoma [86,111,112] and elevated circulating CXCL12 after treatment with VEGFR tyrosine kinase inhibitor cediranib [105,113]. These data suggest that the CXCL12 blockade in combination with other therapies might prevent tumor recurrence and decrease the chemoresistance-induced CXCL12 and further, induce tumor sensitization to other therapies.

9. Concluding Remarks

Taken together, these data support the fact that the CXCL12 pathway is a potential target for the emerging therapies that aim to delay cancer progression, angiogenesis and metastasis, since they may act synergistically with the adjuvant and neoadjuvant anti-tumor treatment. Since data have shown that CXCL12 mediates not only cervical cancer cell migration but also influences overall cancer prognosis, the understanding of tumor biology with focus on CXCL12 will enable the success of therapy currently used and the design of new therapeutic interventions.

Conflict of Interest

All the authors declare no conflicts of interest in this paper.

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