Role of annexin A6 in cancer (Review)

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Abstract. Annexin A6 (AnxA6) is a member of a conserved superfamily of Ca^{2+}-dependent membrane-binding annexin proteins. It participates in membrane and cytoskeleton organization, cholesterol homeostasis, membrane trafficking, cell adhesion and signal transduction. The expression levels of AnxA6 are closely associated with melanoma, cervical cancer, epithelial carcinoma, breast cancer, gastric cancer, prostate cancer, acute lymphoblastic leukemia, chronic myeloid leukemia, large-cell lymphoma and myeloma. AnxA6 exhibits dual functions in cancer, acting either as a tumor suppressor or promoter, depending on the type of cancer and the degree of malignancy. In several types of cancer, AnxA6 acts via Ras, Ras/MAPK and/or FAK/PI3K signaling pathways by mainly mediating PKCα, p120GAP, Bcr-Abl and YY1. In the present review, the roles of AnxA6 in different types of cancer are summarized.

Contents

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1. Introduction

The annexins are highly conserved Ca^{2+}-dependent membrane-binding proteins that display a diverse range of functions in cellular development and differentiation (1-5). They are classified into 5 groups, termed A-E. The annexins of group A, (which includes 12 subgroups, designated as A1-A11 and A13), are present in vertebrates (1).

The annexins are composed of 2 principal domains: A variable N-terminal domain, and a conserved C-terminal core, which contains the Ca^{2+}- and membrane-binding sites (1-5). The C-terminal cores of the annexins are characterized by 4 homologous annexin repeats, with the exception of annexin A6 (AnxA6), which has 8 (1,2). Each repeat contains ~70 highly conserved amino acid residues. These repeats are packed into a disc, which is mostly α-helical, with a slight curvature, and the presence of Ca^{2+} facilitates the binding of membrane phospholipids to the disc region (1). The flexible N-terminal domain is located on the concave side of the annexin. The diversity displayed by this domain is the main criterion for distinguishing the different annexin subfamilies (1,6). Annexins participate in cancer cell proliferation, motility, tumor invasion, metastasis, angiogenesis, apoptosis and drug resistance (7,8).

The human ANXA6 gene is composed of 26 exons, with ~6,000 bp, and is located on chromosome 5q32-q34 (7,8). Unlike the other annexins, AnxA6 contains 2 annexin domains connected by a linker located between the fourth and fifth annexin repeats. These 2 annexin domains may have evolved from fusion duplicates of the ANXA5 and ANXA10 genes (1,2). The Ca^{2+}-binding sites of AnxA6 are located in the annexin repeats 1, 2, 4, 5, 6 and 8 (9). The putative phosphorylation sites of AnxA6 are the residues Ser13, Tyr30 and Thr356 (10). Ca^{2+}-activated AnxA6 binds to negatively charged phospholipids, including phosphatidylserine, phosphatidylinositol and phosphatidic acid (3), and to phosphatidylethanolamine and arachidonic acid (11). The targeting of AnxA6 to membrane locations via the phospholipid is regulated by the pH and the levels of cholesterol (12-14). AnxA6 contributes to membrane organization; participates in cholesterol homeostasis (12,13,15,16); regulates the interactions of membranes and actin during endocytic transport (17,18); and regulates secretory events with S100 proteins (19) and tumor protein D52 (TPD52) (20). The interaction of AnxA6 with activated protein kinase C (PKC)-α

Table I. Negative correlation of AnxA6 expression with various types of cancer.

Tumor type	AnxA6 expression pattern	Implication	Ref.
Melanoma	Low expression levels in melanoma malignancy	AnxA6 acts as a tumor suppressor in melanoma	(26)
Epithelial carcinoma	No expression in A431 cells	Overexpression of AnxA6 leads to growth and tumor suppression effects in A431 cells	(23,27-29)
BC	Low expression levels in invasive ductal carcinoma and mucous adenocarcinoma tissues	AnxA6 is a potential marker for detection of BC and prediction of survival	(28,34,35)
GC	Low expression levels in GC cells and tissues	AnxA6 is a potential suppressor of GC	(30)
PCa	Low expression levels in localized PCa tissues and reduced during PCa progression	AnxA6 inversely correlates with PCa progression	(31,32)
CML	Low expression levels in FDCP-Mix cells on long term exposure to Bcr-Abl PTK	AnxA6 contributes to CML progression regulated by Bcr-Abl PTK	(37)

AnxA6, annexin A6; BC, breast cancer; GC, gastric cancer; PCa, prostate cancer; CML, chronic myeloid leukemia; FDCP, factor-dependent cell progenitors; Bcr, breakpoint cluster region; Abl, Abelson murine leukemia viral oncogene homolog 1; PTK, protein tyrosine kinase.

Table II. Positive correlation of AnxA6 expression with various types of cancer.

Tumor type	AnxA6 expression pattern	Implication	Ref.
CC	High expression levels in SCC	AnxA6 is a potential marker of CC diagnostics and prognosis	(27,31,40)
ALL	High expression levels in samples of patients with ALL and in B-lineage ALL cells	AnxA6 is a candidate marker for monitoring minimal residual disease in B-lineage ALL	(33)
Large-cell lymphoma	High expression levels in mouse high metastatic RAW117 cells	Localized expression of AnxA6 is associated with the metastasis and adhesion of lymphoma cells	(45)
Myeloma	Upregulation of AnxA6 is associated with overexpression of TPD52	AnxA6 promotes tumorigenesis of B-cell malignancies associated with TPD52	(21)

AnxA6, annexin A6; CC, cervical cancer; SCC, squamous cell cervical carcinoma; ALL, lymphoblastic leukemia; TPD52, tumor protein D52.

and p120GTPase-activating protein (GAP) (21-23) may down-regulate the epidermal growth factor receptor (EGFR)/Ras signaling pathway, since p120GAP is the sole GAP known to bind to EGFR and promote the hydrolysis of Ras-GTP (24).

AnxA6 is closely associated with a variety of tumors, as summarized in Tables I and II. It has been implicated as a potential marker for cervical cancer (CC) (25), and as a tumor suppressor in melanoma (26), epithelial carcinoma (23,27-29), breast cancer (BC) (23,28), gastric cancer (GC) (30), prostate cancer (PCa) (31,32) and chronic myeloid leukemia (CML) (33). Furthermore, AnxA6 is considered to act as a promoting factor in the cellular adhesion, motility and invasiveness of BC (34,35), the progression of acute lymphoblastic leukemia (ALL) (36), the adhesion of lymphoma (37), and the secretory processes in myeloma cells (21).

2. Role of AnxA6 in melanoma progression

The incidence of malignant melanoma is increasing worldwide (38). The prognosis is poor if it is not diagnosed at an early stage, and it is responsible for the majority of the mortalities associated with skin cancer (38).

AnxA6 acts as a tumor suppressor in skin cancer, and is involved in the conversion of melanocytes to malignant melanomas. In a study by Francia *et al* (26), the mRNA expression levels of AnxA6 were observed to be lower in murine B16F1O-metastatic melanoma cells than in syngeneic melan-A immortalized melanocyte cells. In addition, the protein levels of AnxA6 were markedly reduced in the B16F1O cells compared with the melan-A cells, and inversely correlated with melanoma progression (26). Immunohistochemical (IHC) analyses demonstrated that the expression of AnxA6 was reduced or eliminated following an increase in tumor malignancy (26). Thus, these results suggest that AnxA6 may inhibit melanoma progression.

3. Roles of AnxA6 in cervical cancer progression and malignancy

CC is the second most common cancer affecting females worldwide (39). Of all the cervical malignancies, 70-80% are squamous cell cervical carcinoma (SCC) originating from squamous cell epithelia (SCEs) (25).

A number of studies have demonstrated that AnxA6 is associated with the progression and malignancy of CC, and it is a potential protein marker for SCC development (25,40). Proteomic results of Lomnytska *et al* (25) demonstrated that AnxA6 was overexpressed in SCC, and the N-terminus of

AnxA6 was upregulated 2-fold in SCC compared with cervical mucosa (CM). In addition, strong expression of AnxA6 was observed in the nucleus of SCC cells, compared with the weak or moderate expression observed in the cytoplasm of SCE cells (25). However, these may be infiltrating immune cells, since their histopathological appearance was observed to be variable (40).

Another study by Lomnytska *et al* (40) indicated that AnxA6 may aid cytology-based diagnostics of SCC precursor lesions, since its expression levels vary during the different sequential steps of SCC carcinogenesis. Their IHC analysis demonstrated the following findings: i) AnxA6 was preferentially expressed in the cytoplasm of SCC cells vs. the cell membranes of SCE; ii) the cytoplasmic expression of AnxA6 increased during the progression from cervical intraepithelial neoplasia 2/3 (CIN2/3) to microinvasive cancer; iii) invasive SCC displayed the highest sensitivity and specificity during IHC detection of AnxA6, whereas microinvasive SCC presented low expression levels of AnxA6 in the cytoplasm; and iv) the number of sporadic AnxA6$^+$ cells among atypical cells increased from CIN2/3 to invasive SCC (40). Based on these results, it is possible to hypothesize that the detectable alterations in the protein expression levels of AnxA6 in SCC precursor lesions may aid in the cytological and pathological diagnostics of CC and its prognostic evaluation. In addition, it has been demonstrated that RNAi-induced knockdown of AnxA6 in human cervix adenocarcinoma HeLa cells enhanced EGF-induced Ras activity and phosphorylation of extracellular-signal-regulated kinases (ERKs)1/2 following EGF stimulation (31). Thus, AnxA6 may function in CC through interactions with the Ras/mitogen-activated protein kinase (MAPK) signaling pathway.

4. Roles of AnxA6 in epithelial carcinoma A431 cells

Human A431 is a model cell line derived from SCE that retains the basic characteristics of the transformed phenotype (27). It displays overexpression of EGFR, increased Ras/MAPK activity (41) and reduced expression or complete supression of endogenous AnxA6 (38).

A study by Theobald *et al* (27) demonstrated that the overexpression of AnxA6 in A431 cells slightly inhibited cell growth when the cells were cultured in high-serum medium, but markedly increased cell proliferation if the serum content was reduced (27). Unlike wild type A431 (wtA431) cells, the AnxA6-expressing A431 cells (A431anx6) reached confluence and stopped proliferating in medium with low serum content, due to contact inhibition (27). Fluorescence-activated cell sorting analysis indicated that the A431anx6 cells were growth arrested in the G_1 phase (27). The inhibition of A431 growth by AnxA6 is considered to involve the EGF-induced Ras signaling pathway, and EGF-induced expression levels of cyclin D1 have been demonstrated to be increased in wtA431, but not A431anx6 cells, which led to markedly reduced clonogenic growth of the A431anx6 cells incubated with EGF compared with the controls (28). *In vivo* evidence has demonstrated tumors formed in nude mice by A431anx6 cells to be >60% smaller than those induced by wtA431 cells (29), highlighting the tumor-suppressor activity of AnxA6 in A431 cells. The expression of AnxA6 in A431 cells may inhibit the EGF-induced Ras signaling pathway by stimulating the Ca^{2+}-dependent membrane recruitment of p120GAP (27-29,41).

Koese *et al* (23) demonstrated that AnxA6 interferes with the growth of A431 cells through interactions with PKCα and EGFR. In A431anx6 cells, the silencing of PKCα markedly increased the colony formation and cell proliferation capacities of A431anx6 cells (23). Furthermore, EGF stimulation was able to induce the upregulation of Thr-654 phosphorylation of EGFR (pT654-EGFR), suppression of Tyr phosphorylation of EGFR (pY-EGFR) and reduction of ERK1/2 phosphorylation, while PKCα depletion decreased pT654-EGFR, increased pY-EGFR and restored EGFR degradation induced by EGF in the A431anx6 cells (23). AnxA6 was recruited for degradation of EGFR in an EGF-inducible manner, and increased levels of AnxA6 promoted the association of PKCα with the cell membrane and the interaction of PKCα with EGFR (23). Regarding the scaffolding function of membrane-anchored AnxA6 for PKCα, AnxA6 promoted PKCα-mediated EGFR inactivation, and negatively regulated downstream signaling for cell growth and proliferation (23). Collectively, these results suggest that AnxA6 displays tumor suppression effects on A431 cells by facilitating the membrane targeting of PKCα and/or p120GAP, which is mediated by downregulation of the EGF-induced Ras activity and reduced expression levels of cyclin D1.

5. Multiple functions of AnxA6 in breast cancer

BC is the most common type of cancer among females. AnxA6 exhibits diverse functions in different BC cell lines and at different invasive stages of BC (42).

AnxA6 contributes to the termination of EGFR-mediated activation of the Ras signaling pathway in BC cells with low expression levels of AnxA6. In a study by Vilá de Muga *et al* (28) AnxA6 was markedly downregulated in EGFR-overexpressed and estrogen receptor (ER)-negative BC cells, including BT20, MDA-MB-468, HBL-100, MDA-MB-231 and MDA-MB-157, compared with the controls. The overexpression of AnxA6 in these BC cells promoted Ca^{2+}- and EGF-induced membrane targeting of p120GAP, and led to a Ca^{2+}-dependent reduction of the EGF-induced activation of Ras and mitogen-activated protein kinase kinase 1/2. The downregulation of AnxA6 in MDA-MB-436 BC cells enhanced colony formation capacity, and increased the EGF-stimulated activity of Pan- and H-Ras (28). The overexpression of AnxA6 in BT20 cells reduced the EGF-induced expression of cyclin D1 (28). Furthermore, reduced levels of pY-EGFR, increased levels of pT654-EGFR and increased membrane association capacity of PKCα were observed in MDA-MB-468 and BT20 BC cells that overexpressed AnxA6 and EGFR (23). The inhibition of growth and proliferation induced by AnxA6 in these cells may occur via its interaction with PKCα and p120GAP, which may lead to reduced Ras signaling.

The protein expression levels of AnxA6 may also be associated with BC invasiveness: Sakwe *et al* (34) demonstrated that AnxA6 was secreted via a Ca^{2+}-dependent exosomal pathway, and the cell surface-associated AnxA6 was observed to be concentrated in the membrane protrusions of BC cells. Other studies indicated that AnxA6 acted as an adhesion receptor to fetuin-A (α2 HS-glycoprotein), a major serum adhesive protein

on the surface of BC cells, by mediating growth signaling (43,44). AnxA6 has been observed to promote the invasiveness of BT-549 BC cells, which express higher levels of AnxA6 than MCF-10A cells, since the depletion of AnxA6 in BT-549 cells reduced their motility and abolished invasiveness (34,35). AnxA6 knockdown in BT-549 BC cells (BT-A6A cells) altered their cell morphology in a dose-dependent manner, compared with parental BT-549 cells (34). When cultured, the parental BT-549 cells developed as cell masses, and were confluent by day 4, while the BT-A6A cells proliferated as single cells in an anchorage-independent mode, and continued to proliferate without attaining confluence (34). These observations suggest that the loss of AnxA6 abolished contact inhibition and enhanced anchorage-independent cell proliferation in BC cells. Furthermore, the expression levels of AnxA6 are associated with the localization of focal adhesions at appropriate plasma membrane sites (34). Sakwe *et al* (34) also demonstrated that depletion of AnxA6 inhibited cell-cell cohesion and cell adhesion/spreading onto specific extracellular matrix components. Immunofluorescence staining of vinculin indicated that the cell-cell and cell-extracellular matrix contact sites in the adhesion plaques of BT-549 cells were peripherally located (34). The activated EGFR was mainly localized at the plasma membrane of BT-549 cells, which expressed high levels of AnxA6, leading to a sustained activation of MAPK ERK1/2. By contrast, in a study by Koumangoye *et al* (35), the activated EGFR was barely detectable at the plasma membranes of HCC1806 and MDA-MB-468 cells, which contained low expression levels of AnxA6, leading to a reduced activation of ERK1/2. Furthermore, the activation of focal adhesion kinase (FAK) and phosphatidylinositol 3 kinase (PI3K) in AnxA6-depleted BT-549 cells was observed to be strongly inhibited (34). Compared with the control BT-549 cells, the levels of EGF-activated EGFR and the EGF-stimulated activation of ERK1/2 and protein kinase B (PKB or Akt) were strongly reduced in AnxA6-depleted BT-549 cells, while the degradation of EGFR was enhanced in these cells (35). In conclusion, AnxA6 participates in enhancing the localization and stabilization of activated EGFR on cell surfaces, and/or enhancing the localization of focal adhesions. The consequently enhanced activation of FAK triggers a sustained activation of downstream effectors that promote the motility and invasiveness of BT-549 BC cells.

Clinically, AnxA6 may be a potential indicator of BC prognosis and invasion stages. A previous study demonstrated that AnxA6 was strongly expressed in normal mammary tissues, while its expression was reduced by ~60 and ~70% in invasive ductal carcinoma and mucous adenocarcinoma tissues, respectively (34). IHC analyses of proliferating cell nuclear antigen indicated that cell proliferation was barely detectable in normal breast tissues, whereas it was more easily detected in invasive ductal carcinoma and mucous adenocarcinoma tissues (34). AnxA6-depleted cells were observed to be more sensitive to the EGFR-targeted tyrosine kinase inhibitors lapatinib and PD153035 than the control BT-549 cells (35). Reduced expression levels of AnxA6 were associated with a better relapse-free survival of patients with BC, while basal levels of AnxA6 were associated with poorer distant metastasis-free survival and overall survival rates of patients with basal-like BC (35). Thus, the expression levels of AnxA6 may aid in the detection of BC, the prediction of survival of patients with basal-like BC and the development of improved EGFR-targeted therapies for BC.

6. AnxA6 is downregulated through promoter methylation in gastric cancer

GC is the second leading cause of cancer-associated mortalities worldwide, and AnxA6 negatively correlates with GC progression (42).

A study by Wang *et al* (30) indicated that the mRNA levels of AnxA6 were downregulated in GC cell lines and primary gastric carcinoma tissues compared with stomach epithelium cells and normal paracancerous tissues, respectively. Restored expression of AnxA6 in MKN28 cells inhibited their growth and clonogenic ability through inhibiting Ras/MAPK activity. The downregulation of AnxA6 in GC cells is a result of the methylation of its promoter region, as indicated by the fact that a typical CpG island (CGI) in the promoter region of the ANXA6 gene was observed to be methylated in a number of GC cell lines. Furthermore, the expression of AnxA6 may be restored following demethylation treatment (30). The methylation of AnxA6 in GC cells was closely associated with yin yang 1 (YY1), a transcription factor involved in the initiation and maintenance of DNA methylation (30). YY1 was bound to AnxA6 through several consensus binding sequences within the promoter region of the ANXA6 gene, and the depletion of YY1 by siRNA reduced the CGI methylation of AnxA6, and consequently restored the expression of AnxA6 (30). The downregulation of AnxA6 by YY1-induced promoter methylation of ANXA6 CGI in GC cells enhanced GC invasiveness and progression through interrupted activity of Ras/MAPK signaling (30). Although the methylation of the ANXA6 promoter CGI was detected in all the 6 GC cell lines studied and in 29 of 156 (18.6%) primary GC tissues, it was not observed to be a significant indicator of the prognosis of patients with GC (30). Therefore, the role of methylation in the promoter of the ANXA6 gene in GC requires further investigation.

7. Potential role of AnxA6 in prostate cancer progression

PCa is the second leading cause of cancer-associated mortalities in males (42). Although prostate-specific antigen (PSA) screening is successful in the early diagnosis of clinically localized PCa, no reliable predictors of the behavior and aggressiveness of PCa have been identified thus far (32).

The mRNA levels of AnxA6 are associated with PCa progression, and the cDNA and mRNA levels of AnxA6 were slightly reduced and significantly downregulated (P=0.0001), respectively, in localized PCa tissues compared with benign ones in a study by Xin *et al* (32). The reduction of AnxA6 was further accentuated during the progression from benign to malignant state in a previous model of PCa (31), implicating the participation of AnxA6 in PCa progression.

8. Roles of AnxA6 in blood cancer

Various types of blood cancer, including leukemia, lymphoma and myeloma, affect the blood, bone marrow and lymph nodes. AnxA6 is involved in B-lineage ALL, CML and large-cell lymphoma (20,33,36,37).

ALL, characterized by excessive lymphoblasts, is a common form of leukemia in children (36). AnxA6 may be a candidate marker for monitoring minimal residual disease in B-lineage ALL. The mRNA levels of AnxA6 were upregulated ≥2-fold in 2 of 4 leukemic samples from patients diagnosed with ALL, compared with normal $CD19^+$, $CD10^+$ B-cell progenitors (36). In addition, high protein levels of AnxA6 were detected in B-lineage ALL cells (36).

CML, a clonal disorder of pluripotent hemopoietic stem cells, is another form of leukemia (33). AnxA6 may contribute to CML progression by association with Bcr-Abl protein tyrosine kinase (PTK) activity. In CML, the constitutively activated hybrid protein Bcr-Abl PTK affects the differentiation and development of primitive hemopoietic progenitor cells. The specific biological consequences of the Bcr-Abl activity in these progenitors were investigated by Pierce *et al* (45) employing a model of the multipotent hemopoietic cell line, termed FDCP-Mix, which was transfected with a temperature-sensitive mutant of Bcr-Abl. The transfected FDCP-Mix cells that were cultured at the optimal temperature for Bcr-Abl PTK activity displayed enhanced survival and proliferation, which mimicked the disease progression in CML by controlling the time of exposure of the FDCP-Mix cells to Bcr-Abl PTK. Previous proteomic results demonstrated that AnxA6 was markedly downregulated (80%) in FDCP-Mix cells following long term exposure to Bcr-Abl PTK, compared with cells that had been exposed to Bcr-Abl PTK for a short time (33). These results were further confirmed by poly(A) polymerase chain reaction analysis of the cDNA and by western blotting (33). Thus, Bcr-Abl interacts with AnxA6 in CML progression.

The cellular localization of AnxA6 is also associated with the metastasis and adhesion of lymphoma cells. Cytofluorographic assays have indicated that AnxA6 is more abundantly expressed on the cell surface of mouse high metastatic RAW117 cells than on RAW117 lymphoma cells (37). AnxA6 may act as a tumor cell-endothelial cell adhesion molecule in lymphoma, as incubation with an anti-AnxA6 antibody markedly inhibited the Ca^{2+}-dependent adhesion of RAW117 to endothelial cells (37). Collectively, the upregulation and relocalization of AnxA6 on the cell surface may promote the progression and metastasis of lymphoma.

Additionally, AnxA6 may promote the tumorigenesis of B-cell malignancies in association with TPD52. It has been previously observed that the overexpression of TPD52 contributes to the progression of BC, PCa and ovarian cancer (OC) (46-48), and acts as a marker to differentiate B cells from plasma cells (21). This hypothesis is supported by the fact that AnxA6 was coimmunoprecipitated with TPD52 in the Thiel human myeloma cell line in a Ca^{2+}-dependent manner (21). Thus, the upregulation of AnxA6 in response to overexpression of TPD52 may enhance the tumorigenesis of B-cell malignancies.

9. Conclusion

The disregulation of AnxA6 has been demonstrated to be involved in melanoma, CC, epithelial carcinoma, BC, GC, PCa, ALL, CML, large-cell lymphoma and myeloma, as indicated in Tables I and II. Thus, AnxA6 may be a potential biomarker for the diagnosis, treatment and prognosis of certain tumors. AnxA6 displays tumor suppressor effects in melanoma, epithelial carcinoma, GC, PCa and CML. Its downregulation promotes the development and enhances the invasiveness and metastasis of these types of cancer. AnxA6 exhibits tumor-potentiating effects in CC, ALL, large-cell lymphoma and myeloma. The loss of AnxA6 suppresses the invasiveness and motility of BC and BC cells, while enhancing the anchorage-independent cell growth of BC cells. The specific role of AnxA6 depends on the type of cancer and the level of malignancy. Thus far, the action of AnxA6 has been most commonly associated with the deregulation of Ras, Ras/MAPK and FAK/PI3K signaling activities, mainly through the interactions with PKCα, p120GAP, Bcr-Abl and YY1. Regardless, the roles of Anxa6 in various types of cancer and the details of its mechanism of action require further study.

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