

## The role of annexin A4 in cancer

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### 1. ABSTRACT

Annexin A4 (ANXA4) is a member of the annexin family that binds to both calcium ions and phospholipids. Studies indicate that ANXA4 modulates membrane permeability and membrane trafficking, participates in cellular growth and apoptosis, enhances tumor invasion and promotes anti-tumor drug resistance. The overexpression of ANXA4 has been identified in various clinical epithelial tumors including: lung, gastric, colorectal, pancreatic, gallbladder, breast, renal, ovarian, laryngeal, and prostate cancers. In addition, upregulation and nuclear translocation of ANXA4 have been observed in the progression of colorectal cancer and ovarian serous carcinoma. Knockdown of ANXA4 attenuated migration in ovarian cancer and breast cancer cells. In contrast, knockdown of ANXA4 increased susceptibility to platinum in ovarian cancer and malignant mesothelioma cells. It is conceivable that ANXA4 is an indicator for tumor development, invasion, chemo-resistance, poor outcomes of cancer patients, and may be a potential target for therapeutic intervention.

### 2. INTRODUCTION

Annexin A4 (ANXA4) is a member of the annexin family. Annexins belong to a conserved

multi-gene family composed of calcium ions ( $\text{Ca}^{2+}$ ) and phospholipid binding proteins. Annexins can be classified into five groups (A-E) (1). Group A is found in vertebrates (including annexin A1-11 and A13). Groups B, C, D and E are found in invertebrates, fungi/mold, plants, and protists.

Biochemically, annexins share two essential characteristics in structures and functions. The basic structure of annexins consists of 2 major domains. A conserved domain at the C terminus includes 4 annexin repeats, each of which is about 70 amino acid residues in length. A unique N-terminal domain composed of 20-200 amino acid residues was identified. The N-terminal domain decides the subgroup in the annexin family and the function of individual annexin *in vivo*. The four annexin repeats form a highly  $\alpha$ -helical structure and represent the canonical  $\text{Ca}^{2+}$  regulated membrane binding module, which can produce reversible  $\text{Ca}^{2+}$ /phospholipid binding proteins. In addition, various atypical  $\text{Ca}^{2+}$ -independent lipid binding properties are found among different annexins. The N-terminal domain of annexin contributes to the diversity of the annexin family and regulates the interactions between protein ligands and

annexin-membrane association. The interactions and associations within annexin are not fully understood.

The variations in the expression or location of annexins may cause pathological changes that can lead to different disease states (1-4). Annexins have diverse functions that include endocytosis, exocytosis, cell division, apoptosis, growth regulation, and inflammation. The dysregulated expression of annexins is potentially associated with disorders in the heart and circulation, physiological stress, diabetes, and neoplastic processes. Abnormal level of annexins have been found in a range of tumors. Although annexins have not been proven to be directly correlated with any single tumor, accumulated evidence shows that annexins may be of high value as molecular targets in the study of neoplastic development and progression.

### 3. STRUCTURES AND FUNCTIONS OF ANXA4

The ANXA4 gene is located on human chromosome 2q13. ANXA4 is a 36-kDa protein that aggregates on cell membranes and contains four annexin repeats with one  $\text{Ca}^{2+}$ -binding site and five  $\alpha$ -helices in each repeat, which can bind phospholipids in a calcium-dependent manner. The N-terminal domain of ANXA4 is 12 amino acids in length. In addition, the trigonal crystal form of N-terminally truncated ANXA4, first determined by Zanotti and colleagues, is highly homologous to the other annexins in the same family, especially annexin A5 (5). The propensity of annexins A4 and A5 is to form trigonal crystals in ordered two-dimensional arrays on membrane surfaces. The trimetric complex presents as a functional unit (6, 7). Though the underlying mechanism is not clear, ANXA4 plays an important role in modulating membrane permeability, membrane trafficking, cellular growth, and apoptosis of epithelial cells. ANXA4 phosphorylation by protein kinase C (PKC) has been shown to inhibit vesicle trafficking (6). ANXA4 was identified as a novel candidate as substrates of the mammalian target rapamycin complex 1 (mTORC1) and caspase-3, (8, 9) respectively, The central role of mTORC1 is to regulate cellular growth when caspase-3 induces apoptosis. In addition, studies indicate that ANXA4 differentially modulates the NF- $\kappa$ B signaling pathway, depending on its interactions with p50 subunit and the intracellular  $\text{Ca}^{2+}$  ion level (10). Jeon *et al.* discovered that ANXA4 interacts with the p50 subunit of NF- $\kappa$ B and overexpression of ANXA4 suppresses transcriptional activity of NF- $\kappa$ B. However, in the presence of high  $\text{Ca}^{2+}$  levels, enhanced interactions between ANXA4 and the p50 subunit were observed with the consequent upregulation of transcriptional activity of NF- $\kappa$ B and co-translocation of ANXA4 and p50 to the nucleus.

### 4. ANXA4 AND CANCERS

ANXA4 is expressed exclusively in epithelial cells. Increased expression of ANXA4 has been reported

in various clinical epithelial tumors including lung, gastric, colorectal, pancreatic, gallbladder, breast, renal, ovarian, laryngeal, endometrial and prostate cancer (11-21). The study of ANXA4 may help early diagnosis and get a better understanding of the molecular mechanisms in cancer progression.

#### 4.1. ANXA4 and gastric cancer

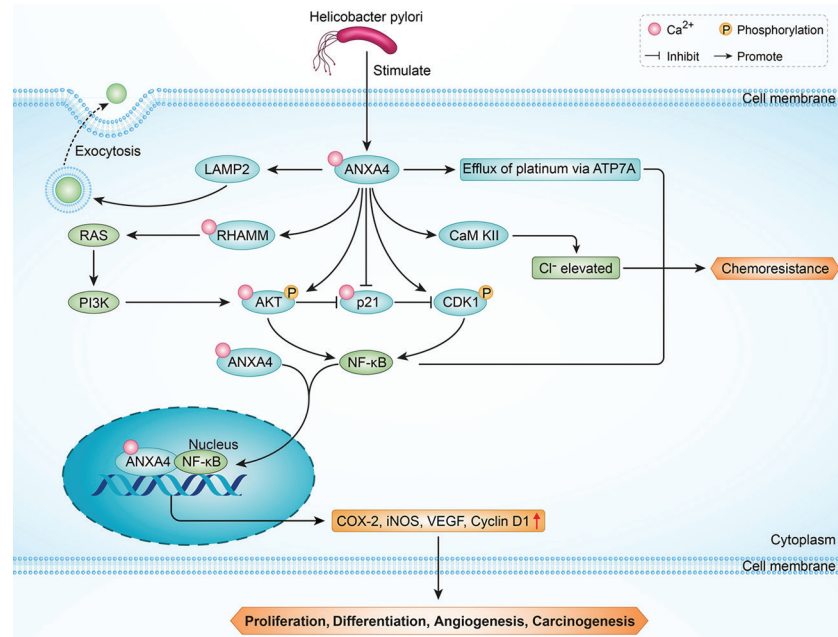
ANXA4 is mainly expressed in the glandular epithelium of the stomach (22). Lin *et al.* reported the relationship between ANXA4, helicobacter pylorus, and gastric cancer. Using 2-DE and Western blotting, ANXA4 was found to be overexpressed in gastric cancer tissues affected by helicobacter pylori infection compared with that in normal tissues and tumor tissues without helicobacter pylori infection (23). To identify the specific region with ANXA4 expression on tissue samples from gastric cancer patients with or without helicobacter pylori infection, immunohistochemical stains with anti-ANXA4 antibody were performed, revealing that ANXA4 was only present in nuclear and cytoplasmic stains in the tumor tissue with helicobacter pylori infection. Subsequent studies showed ANXA4 expression was elevated in SCM-1 cells co-cultured with helicobacter pylori in a dose-response manner (23).

The following studies showed ectopic expression of ANXA4 promoted gastric cancer cell proliferation and increased the production of hyaluronan mediated motility receptor (RHAMM) and lysosomal-associated membrane protein 2 (LAMP2). Moreover, ANXA4 can regulate downstream signals, including the activation of AKT, cyclin-dependent kinase 1 (CDK1), and the suppression of p21 in a  $\text{Ca}^{2+}$ -assisted manner. RHAMM is overexpressed in several cancers including gastric cancer and ovarian clear cell carcinoma, and participates in cell signaling, cell proliferation, tumor development, RAS signaling cascade, and activation of AKT (24-26). Activation of AKT, which could be promoted by helicobacter pylori, was reported to be a biomarker in gastric cancer progress, thus inducing the RAS signaling cascade to transduce downstream signals and suppress apoptosis by stimulating NF- $\kappa$ B (10, 27, 28). CDK1-cyclin B1 complexes can regulate the cell cycle G2/M phase and have been reported to be implicated in promoting carcinogenesis (29). Additionally, elevated expressions of CDK1 are associated with the progression of helicobacter pylori induced gastritis to mucosa-associated lymphoid tissue lymphoma (30). The inhibitions of CDKs can suppress NF- $\kappa$ B pathways (31) (Figure 1).

Taken together, we can conclude that ANXA4 may be involved in tumor development in gastric cancer patients with helicobacter pylori infection through activating relevant downstream signals including RHAMM, LAMP2, AKT, CDK1, p21, and NF- $\kappa$ B in a  $\text{Ca}^{2+}$ -assisted manner.

#### 4.2. ANXA4 and ovarian clear cell cancer

The ANXA4 gene was the signature gene of ovarian clear cell carcinoma (CCC), which was first



**Figure 1.** Schematic representation of the ANXA4-associated molecular mechanism on cancer. ANXA4 binds to the plasma membrane in a calcium- dependent manner and regulates its downstream signaling transduction. ANXA4 up-regulates LAMP2, a lysosomal marker involved in exocytosis, and RHAMM, which activates RAS and PI3K. The up-regulation of these two markers subsequently leads to activation of AKT. ANXA4 up-regulates AKT and CDK1 activation. In addition it down-regulates p21 in a calcium-assisted manner, eventually leading to activation of NF-κB. In high levels of Ca<sup>2+</sup>, ANXA4 and the p50 subunit of NF-κB co-translate to the nucleus, subsequently enhancing expression of COX-2, iNOS, VEGF and cyclin D1, and resulting in proliferation, differentiation, angiogenesis and carcinogenesis. Possible mechanisms for ANXA4-induced chemo-resistance include increased efflux of cellular platinum via the copper transporter ATP7A, modulation of NF-κB transcriptional activity, and chloride-and-calcium dependent manner.

identified by Schwartz *et al* (32). Subsequent studies have found that the expressions of downstream signals including ANXA4, RHAMM and LAMP2 are elevated in ovarian CCC cell lines using two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE) (33, 34). In a recent study, significant growth suppression with ANXA4 knockdown (KO) in both OVTOKO and OVICE cell lines were observed. Additionally, ANXA4 KO-OVICE cells showed significantly decreased migration and invasion capability (21). These results suggest that ANXA4 may play an important role in carcinogenesis and progression of ovarian clear cell cancer.

#### 4.3. ANXA4 and ovarian serous carcinoma

ANXA4 protein was upregulated in ovarian serous carcinoma specimens, and patients with nuclear translocation of ANXA4 had a worse survival rate than those without nuclear translocation, suggesting that overexpression and nuclear translocation of ANXA4 may be implicated in chemo-resistance and poor outcomes of ovarian serous carcinoma patients (35). However, another study performed by Kim *et al.* did not reveal ANXA4 as a significant prognostic factor in ovarian serous carcinoma (36). Further study on the relationship between ANXA4 and ovarian serous carcinoma is urgently needed to ascertain the precise effect of ANXA4 in ovarian serous carcinoma.

#### 4.4. ANXA4 and renal clear carcinoma

Among the annexin family, ANXA4 has the highest expression levels in kidney. During embryonic development, ANXA4 plays an undefined role in renal organogenesis. However, the detailed mechanism remains unknown (37, 38). Using two-dimensional gel electrophoresis and mass spectrometry, increased expression of ANXA4 was identified in conventional renal clear carcinoma (RCC) tissue as compared with patient-matched normal kidney cortex tissue (39). Similar results were reported that the expression of ANXA4 was increased in RCC tissue compared with non-neoplastic kidney tissue using two-dimensional polyacrylamide gel electrophoresis and verified by RT-PCR (15). In addition, an altered localization of ANXA4 was observed in tumor cells compared with normal tissues in renal clear carcinoma patients (15). Taken together, ANXA4 may play an important role in RCC.

#### 4.5. ANXA4 and breast cancer

Breast cancer is the second leading cause of cancer-related deaths and the most common malignant tumor found in women (40). Although advanced treatments have been introduced, breast cancer is a leading cause of female mortality. In an effort to improve prognoses, studies on related molecular mechanisms of breast cancer is of essence. ANXA4 was expressed

in ductal and lobular glandular epithelium of breast with different intensity (22). Though Haiman *et al.* did not locate ANXA4 as a risk gene studies for breast cancer with genome-wide association (41), ANXA4 was found to be overexpressed in human breast cancer tissues using a proteomic approach by Deng *et al.* (14). *In vitro* studies found ectopic expression of ANXA4 promoted the migration of human breast cancer MCF-7 cell (15). Hence, additional studies regarding the association of ANXA4 and breast cancer are warranted.

### 4.6. ANXA4 and colorectal cancer

ANXA4 is highly expressed in the glandular epithelium from normal intestine including duodenum, jejunum, ileum, appendix, colon and rectum (22). ANXA4 was identified as a potential biomarker for colorectal cancer through two-dimensional differential gel electrophoresis (2D-DIGE) in several studies (13, 42, 43). Subsequently, a clear staining of ANXA4 in epithelial cells in colorectal cancer tissues, especially in basolateral membranes was reported. Also, upregulation of ANXA4 and translocation of ANXA4 to the cytoplasm and nucleus were observed in tissue specimens of colorectal cancer patients with advanced stage in disease, suggesting that ANXA4 is associated with colorectal tumor dissemination. This is consistent with previous studies in breast cancer cells (15). Similarly, Duncan *et al.* reported the upregulation of ANXA4 was associated with advanced tumor stage and decreased survival (12). Taken together, we can postulate that ANXA4 is associated with colorectal cancer, possibly playing a role in tumor progression.

### 4.7. ANXA4 and pancreatic cancer

ANXA4 is highly expressed in centroacinar cells and the ductal system in the pancreas, but not in acinar cells (22). 2D-PAGE in combination with mass spectrometry (MS) showed that ANXA4 was overexpressed in tumor tissues compared with normal and pancreatitis tissues whereas annexin A5 was downregulated (19). Similarly, another study also reported that ANXA4 was overexpressed in golden hamster pancreatic cancer tissue samples (14). However, Sitek *et al.* reported that ANXA4 expression in pancreatic intraepithelial neoplasia and carcinoma tissues were significantly lower than that in normal pancreatic epithelium. ANXA4 had the lowest levels of expression in carcinoma tissues, suggesting that ANXA4 is downregulated during pancreatic tumor progression (44, 45). Taken together, the definite effect of ANXA4 in pancreatic cancer needs to be further elucidated.

### 4.8. ANXA4 and gallbladder cancer

ANXA4 and annexin A2 are expressed in bile duct and gallbladder epithelium (22). In our previous study, proteomic data identified overexpressed ANXA4 in primary gallbladder cancer compared with cholecystitis and normal gallbladder tissue specimens (18). Furthermore, in our own preliminary studies (unpublished

data), knockdown of ANXA4 using shRNA transfection significantly inhibited gallbladder cell growth, migration, and invasion in GBC-SD and NOZ cells. Knockdown of ANXA4 significantly reduced NF- $\kappa$ B transcriptional activity and inhibited relevant downstream signals including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), cyclin D1, and vascular endothelial growth factor (VEGF) (Figure 1). Taken together, ANXA4 could be a novel biomarker and a potential therapeutic target for primary gallbladder cancer.

### 4.9. ANXA4 and other cancers

In prostate cancer, Haiman *et al.* identified ANXA4 as the possible associated gene through genome-wide association studies. However, ANXA4 was not significant when evaluated with the Bonferroni criteria (41). Proteomics and immunohistochemistry showed that ANXA4 was elevated in serous endometrial cancer and laryngeal carcinoma tissues (14, 20). As ANXA4 has been reported to be involved in several cancers including gastric cancer and ovarian cancer, more relevant studies are needed to confirm the specific mechanism of ANXA4 in prostate, laryngeal and endometrial cancer.

## 5. ANXA4-MEDIATED CHEMO-RESISTANCE

ANXA4 is potentially involved in chemo-resistance in several cancers including ovarian cancer, lung cancer, and malignant mesothelioma. Kim *et al.* revealed that ANXA4-transfected ovarian non-CCC cells showed significantly stronger resistance to carboplatin compared with control cells (36). In studies, knockdown of ANXA4 resulted in greater sensitivity to carboplatin in OVTOKO cells (21). Additionally, ANXA4 conferred platinum-resistance in ovarian serous cancer. Levels of ANXA4 were significantly higher in chemo-resistant cancer tissues compared with chemo-sensitive cancer tissues of ovarian serous cancer. Ovarian serous cancer patients with nuclear localization of ANXA4 showed significantly higher resistance to platinum (35). Similarly, it was reported that ANXA4 participated in paclitaxel-resistance of lung cancer. Increased expression of ANXA4 was detected in a paclitaxel-resistant lung H460 cell line. Immunostaining with ANXA4 antibody showed that ANXA4 was found to be primarily located in the nucleus of paclitaxel-resistant H460 cells. Furthermore, short-term stimulation of 10 nM paclitaxel in H460 cells caused the induction of ANXA4 as well as increased resistance to paclitaxel (46). In addition, ANXA4 was also implicated in cisplatin-resistance in malignant mesothelioma (47). High levels of ANXA4 were detected in cisplatin-resistant mesothelioma cells and human clinical malignant mesothelioma tissues. Moreover, knockdown of ANXA4 gene increased the susceptibility to cisplatin whereas transfection of ANXA4 showed the opposite effect. Increased evidence of the important role of ANXA4 in cancer chemo-resistance has prompted

investigations in the mechanisms of ANXA4-induced chemo-resistance (Figure 1).

### 5.1. Increased cellular efflux of platinum via the copper transporter ATP7A

In previous studies, Kim *et al.* demonstrated that overexpression of ANXA4 confers chemo-resistance to carboplatin using ANXA4-transfected ovarian serous adenocarcinoma (SAC) cells (36). To investigate the mechanism of ANXA4-induced chemo-resistance, they quantified the intracellular platinum concentration after treatment of OVSAHO/ANXA4 cells and control cells with carboplatin. Significantly reduced accumulation of intracellular platinum was noted in OVSAHO/ANXA4 cells compared with control cells, indicating that ANXA4 inhibited cellular platinum influx and/or increased cellular platinum efflux. To ascertain the effect of ANXA4 in influx or efflux, the results for 0 and 360 minutes of carboplatin-free incubation were compared. The results revealed that ANXA4 promoted increased cellular platinum efflux in OVSAHO/ANXA4 cells compared with controls. In agreement with this result, Morimoto *et al.* found that platinum accumulation was significantly reduced in ANXA4 overexpressed cells or ANXA4 deletion mutants compared with control cells (48). Further studies investigated the precise mechanisms underlying the relationship of ANXA4 to platinum resistance. Matsuzaki *et al.* revealed that ANXA4 relocated from the cytoplasm to the cellular membrane after exposure to platinum drugs where it was subsequently co-localized with the copper transporter ATP7A. Suppression of ATP7A by small interfering RNA in ANXA4-overexpressed platinum-resistant cells increased the sensitivity to platinum drugs compared with control cells (49). Taken together, it is conceivable that ANXA4 confers chemo-resistance in part by promoting cellular platinum efflux mediated by the copper transporter ATP7A.

### 5.2. Modulation of NF- $\kappa$ B transcriptional activity

NF- $\kappa$ B has been reported to play important roles in cancer development, cancer progression and chemo-resistance (50, 51). A recent study reported that ANXA4 differentially modulates the NF- $\kappa$ B transcriptional activity, depending on its interactions with the p50 subunit and the intracellular calcium ion level (10). In subsequent studies, they investigated the altered localizations of p50, p65, and ANXA4 after etoposide treatment. Results showed that ANXA4 cotranslated to the nucleus with the p50 subunit after etoposide treatment, and enhance subsequent transcriptional activation of NF- $\kappa$ B. Therefore, the resistance to etoposide-associated apoptosis was induced. Although NF- $\kappa$ B has been demonstrated to play key roles in chemo-resistance of various cancers, the synergistic effect with ANXA4 needs further investigations.

### 5.3. Chloride-and-calcium dependent manner

Recently, Morimoto *et al.* proposed a novel mechanism of ANXA4-induced chemo-resistance that

the calcium-binding site in annexin repeats induces resistance to the platinum-based drug through elevating the intracellular chloride concentration in RMG-I cells (48). Studies revealed that decreased intracellular platinum concentration was associated with annexin repeat domain. To assess whether the calcium-binding site in the annexin repeat participated in the chemo-resistance, they detected the platinum content in cells with or without annexin repeats after cisplatin-free incubation, which suggested the calcium-binding site is responsible for platinum-resistance. To elucidate the molecular mechanism of ANXA4-associated resistance, they monitored Cl<sup>-</sup> content in cells overexpressing full-length ANXA4, two deletion mutants, and control cells. Subsequent results showed the inverse ratio of fluorescence 1/(F30/F0) was significantly increased in platinum-resistant cells in comparison with platinum-sensitive cells. Consistent with this study, elevated levels of Cl<sup>-</sup> were also observed in cisplatin-resistant cells compared with cisplatin-sensitive cells. Furthermore, intracellular cisplatin content showed the opposite effect in a previous study (52). Therefore, we can postulate that ANXA4 induces platinum-resistance partly in chloride-and-calcium manner.

The evidences suggested mechanisms of ANXA4-induced chemo-resistance were mainly non-specific to a range of anti-cancer drugs rather than drug-specific. Therefore, inhibition of ANXA4 expressions can induce cancer cell susceptibility to various anti-cancer drugs, and further investigations are needed to fully understand the signaling pathway related to ANXA4-associated chemo-resistance.

## 6. CONCLUSIONS

ANXA4 is upregulated in tumor tissues as mentioned above, and translocation of ANXA4 to the cytoplasm and nucleus are associated with advanced tumor stage in renal clear cell carcinoma and colorectal cancer. Furthermore, ANXA4 has been implicated in chemo-resistance in ovarian cancer, lung cancer, and malignant mesothelioma. Taken together, these studies highlight an emerging role of ANXA4 as a potential target in cancer progression and chemo-resistance. Further studies are warranted to establish the efficacy of ANXA4 targeted therapy in relevant with cancer patients.

## 7. ACKNOWLEDGEMENTS

Houshan Yao and Chang Sun contribute equally to this work and they are the co-first authors, Zhiqian Hu (Department of General Surgery, Shanghai Chang Zheng Hospital, Second Military Medical University, 415 Feng Yang Road, Shanghai 200003, China, Tel: 86-21-81885596, Fax: 86-21-81885591) and Weijun Wang are the co-corresponding authors. This work was supported by the grant from the National Natural Science

Foundation of China (81201941) and the grant from the Natural Science Foundation of Shanghai (12ZR1454800).

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**Abbreviations:** ANXA4: Annexin A4; Ca<sup>2+</sup>: calcium ions; PKC: protein kinase C; mTORC1: mammalian target rapamycin complex 1; RHAMM: hyaluronan mediated motility receptor; LAMP2: lysosomal-associated membrane protein 2; CDK1: cyclin-dependent kinase 1; CCC: clear cell carcinoma; 2D-PAGE: two-dimensional-polyacrylamide gel electrophoresis; KO: knockdown; RCC: renal clear carcinoma; 2G-DIGE: two-dimensional differential gel electrophoresis; MS: mass spectrometry; COX-2: cyclooxygenase-2; iNOS: inducible nitric oxide synthase; VEGF: vascular endothelial growth factor; SAC: serous adenocarcinoma

**Key Words:** Annexin A4, Cancer, Chemo-Resistance, Review

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