Targeting metabolic flexibility via angiopoietin-like 4 protein sensitizes metastatic cancer cells to chemotherapy drugs

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Abstract

Overcoming multidrug resistance has always been a major challenge in cancer treatment. Recent evidence suggested epithelial-mesenchymal transition plays a role in MDR, but the mechanism behind this link remains unclear. We found that the expression of multiple ABC transporters was elevated in concordance with an increased drug efflux in cancer cells during EMT. The metastasis-related angiopoietin-like 4 (ANGPTL4) elevates cellular ATP to transcriptionally upregulate ABC transporters expression via the Myc and NF-κB signaling pathways. ANGPTL4 deficiency reduced IC50 of anti-tumor drugs and enhanced apoptosis of cancer cells. In vivo suppression of ANGPTL4 led to higher accumulation of cisplatin-DNA adducts in primary and metastasized tumors, and a reduced metastatic tumor load. ANGPTL4 empowered cancer cells metabolic flexibility during EMT, securing ample cellular energy that fuels multiple ABC transporters to confer EMT-mediated chemoresistance. It suggests that metabolic strategies aimed at suppressing ABC transporters along with energy deprivation of EMT cancer cells may overcome drug resistance.

Keywords: Epithelial-mesenchymal transition, Multi-drug resistance, Angiopoietin-like 4, ATP-binding cassette transporters

Main text

Cytotoxic chemotherapy is one of the mainstays of cancer treatment. Despite being an important therapeutic option for most cancer patients, the development of multiple drug resistance (MDR) by tumors has emerged as a major obstacle that limits the efficacy of chemotherapy [1]. Recent evidence also indicates that epithelial-mesenchymal transition (EMT) plays a role in MDR [2, 3]. Although these studies have oversimplified the relationship between EMT and MDR, they highlight a need for a better understanding of these two complex and poorly understood processes which often co-exist clinically.

A well-established cause of MDR is the increased expression of ATP-binding cassette (ABC) transporters, that efflux various chemotherapeutic compounds from cells [4]. Their broad specificity has been the subject of numerous attempts. However, the results of clinical trials have been rather disappointing. The failure may be attributed to the lack of specificity, resulting in toxicity and adverse drug interaction, or singularly targeting one transporter. Increased expression of ABC transporters necessitates a concomitant increase in cellular adenylate energy to fuel their activities, otherwise the cancer cells will experience an ‘ATP debt.’ Thus, targeting cancer metabolism has emerged as a promising strategy. However, the metabolic flexibility shown by cancer cells during EMT poses significant therapeutic challenges. In this context, the role of angiopoietin-like 4 (ANGPTL4) as a driver of EMT-enriched metabolic changes is a prime target. Numerous clinical and molecular evidence have...
Fig. 1 (See legend on next page.)
identified ANGPTL4 as a pro-metastatic gene \[5, 6\]. Recent studies showed that ANGPTL4 augmented cellular metabolic activity and coordinated the energy demands required for EMT competency \[7, 8\].

In this study, we explore the attenuation of metabolic flexibility as a potential strategy to attenuate the activities of ABC transporters and to overcome MDR in metastatic cancer cells.

**Results and discussion**

**ANGPTL4 elevates cellular ATP to fuel ABC transporters in cancer cells during EMT**

We examine the expression of ABC transporters in three in vitro EMT models using the polarized gastric carcinoma line MKN74 \[7\]. EMT was initiated by either hypoxia (1% O\textsubscript{2}) or TGFβ1. EMT was initiated in MKN74\textsubscript{Snai1-ER}, a MKN74 line carrying a Snai1-ER transgene, by 4-hydroxytamoxifen (4-OHT). Upon exposure to stimuli, the MKN74 cells underwent EMT after 48–96 h as confirmed by immunoblotting and qPCR of epithelial- and mesenchymal-associated genes (Additional file 1: Figure S1A–C).

Our focussed gene expression analysis revealed an enrichment of multiple ABC transporters genes, including ABCB1 (MDR1), ABCC1 (MRP1) and ABCG2 (BCRP), across the EMT models (Fig. 1a). Flow cytometry confirmed elevated expression of several ABC transporters during EMT of MKN74 and MCF-7 cancer cells (Fig. 1b and Additional file 1: Figure S1D). Regardless of the stimuli, cancer cells undergoing EMT showed a higher drug efflux capacity as evidenced by a 30–50% decrease in intracellular fluorescent dye (Fig. 1c). Next, the relative contribution of ABCB1, ABCC1 and ABCG2 were determined by using inhibitor Verapamil, MK-571, and Novobiocin, respectively. Our finding highlighted the significance of ABCC1 and ABCG2 in MDR during EMT (Fig. 1d).

ANGPTL4 is a critical player that coordinate cellular metabolic activities that enhances metastasis \[7\]. We observed a higher ABCB1 protein during EMT of multiple cancer cell lines and stage-specific human tumor biopsies that were positively correlated with cANGPTL4, the C-terminal fragment of ANGPTL4 (Additional file 1: Figure S2A–C). Interrogation of the microarray data (GSE71280), revealed an enrichment of genes regulating glucose, fatty acids and amino acid metabolisms, autophagy and ROS metabolism (Fig. 1e). The suppression of ANGPTL4 resulted in significant alteration of > 50% of genes identified in our microarray analysis, suggesting that ANGPTL4 plays a pivotal role on metabolic flexibility during EMT (Additional file 1: Figure S3A). Consistent with the microarray analysis, the immunoneutralization (α-cANGPTL4) or siRNA knockdown (ΔANGPTL4) of ANGPTL4 reduced 2-NBDG uptake, a fluorescent glucose analog (Additional file 1: Figure S3B) and diminished glycolysis (Fig. 1f). Similar observation was obtained using the MKN74\textsubscript{Snai1ER-shANGPTL4} Cells (Additional file 1: Figure S3C), MKN74\textsubscript{Snai1ER} harboring doxycycline (dox)-inducible shANGPTL4 transgene. Conversely, recombinant cANGPTL4 (rh-cANGPTL4) treatment increased glycolysis (Fig. 1f). The β-oxidation of long-chain fatty acids was also elevated during TGFβ1-induced EMT, which was abolished by ANGPTL4-knockdown and restored by exogenous rh-cANGPTL4 (Fig. 1g). Furthermore, the cellular ATP concentration was elevated during EMT and reduced upon ANGPTL4 depletion (Additional file 1: Figure S3D–E).

Drug efflux by ABC transporters is an ATP-dependent process. We observed higher fluorescent dye was trapped intracellularly and less actively pumped out in ANGPTL4-depleted cells (Additional file 1: Figure S3F–G). Notably, the IC\textsubscript{50} of cisplatin and doxorubicin was reduced by ~ 50% in MKN74\textsubscript{ΔANGPTL4} than MKN74\textsubscript{CTRL} (Fig. 1h). Altogether, ANGPTL4-mediated metabolic flexibility helps cancer cells secure cellular ATP for EMT-dependent drug efflux by ABC transporters.

**ANGPTL4 deficiency in metastatic cancer cells impairs cisplatin efflux in vivo**

To confirm the above observations in vivo, we examined the metastasis of MKN74\textsubscript{Snai1ER-shANGPTL4} cells in response to cisplatin in the presence or absence of ANGPTL4 (Fig. 2a). Dox-diet (4-OHT:dox) suppressed ANGPTL4 and attenuated primary xenograft growth...
**Fig. 2** (See legend on next page.)
compared with chow-diet (4-OHT:chow)-derived xenografts (Fig. 2a-b). In 4-OHT:dox xenografts, ~ 91% of cytokeratin18 (CK18)-positive cancer cells has cisplatin-DNA adducts compared with ~78% in 4-OHT:chow xenografts (Fig. 2c). Importantly, 4-OHT:dox xenografts have higher apoptotic cancer cells than 4-OHT:chow xenografts (Fig. 2c). The 4-OHT:dox tumors showed ~ 91% of cytoplasm+ cells among human CK18 + MKN74 cells that metastasized to the lung tissues of 4-OHT:chow and 4-OHT:dox mice. Analysis showed 38.6% (red) CK18-positive cells in 4-OHT:chow lungs compared with 28.61% (red) 4-OHT:dox lungs. Data are represented as mean ± s.d. from n = 3 independent experiments. *P < 0.05, **P < 0.01

Coordinated upregulation of multiple ABC transporters by c-Myc and NF-κB

The rh-cANGPTL4 upregulated the mRNA levels of seven ABC transporters (Additional file 1: Figure S4A). Following a kinase inhibitor array screen and IPA analysis (Additional file 1: Figure S4B-C), we deciphered that cANGPTL4 activated key signaling mediators of the PI3K/AKT, NF-κB and MEK/ERK pathways and culminated in c-Myc and NF-κB activation (Fig. 3a). Our immunoblot results confirmed the phospho-activation of these kinases and transcription factors in response to rh-cANGPTL4 stimulation (Fig. 3b). In silico analysis of the regulatory promoter regions of these seven ABC transporters [9] identified many putative c-Myc and NF-κB transcription factor binding sites (Fig. 3c, Additional file 1: Table S1). Further investigation showed that rh-cANGPTL4 upregulated ABC transporters which were attenuated by siMYC or IKK2 Inhibitor IV (Fig. 3d and Additional file 1: Figure S4A). Flow cytometry confirmed the elevated expression of ABCB1, ABCG1 and ABCG2 in rh-cANGPTL4-treated MKN74, whose levels were reduced when c-Myc and NF-κB activities were impaired (Fig. 3d). Quantitative chIP analysis on MKN74 treated with rh-cANGPTL4 revealed differential transcriptional regulation of the ABC transporters genes by c-Myc and NF-κB activated by rh-cANGPTL4 (Fig. 3c). The specificity and efficiency of PCR were verified by melt curve analysis (Additional file 1: Figure S5).

We probe the TCGA database to reveal the potential clinical relevance of ANGPTL4 (Additional file 1: Figure S6). Twenty one different tissue sites out of 61 primary sites in TCGA, i.e. 34% of human tumor types, showed alteration in ANGPTL4, with higher representation in uterus, skin and bladder cancers (Additional file 1: Figure S6A). ANGPTL4 has some predictive value in survival prediction due to its high correlation with c-Myc and NF-κB (Additional file 1: Figure S6B-C). Many other factors can explain the survivability of these cases, among which is the post-translational processing of ANGPTL4 to cANGPTL4, which is responsible for the pro-oncogenic action of ANGPTL4.

In summary, our research showed that cancer cells undergoing EMT exhibited metabolic flexibility that secure ample cellular adeny late energy to fuel the increased activities of ABC transporters. Importantly, such re-wiring of metabolic dependency circumvents metabolic therapies designed to target individual metabolic pathways [10]. Our findings underscore the usefulness of metabolic strategies aimed at suppressing ABC transporters along with energy deprivation of EMT cancer cells can overcome drug resistance in metastatic cancer cells. Additionally, the reduction in chemoresistance when anti-ANGPTL4 is used concurrently with conventional chemotherapy agents has the potential to prolong the efficacy of conventional chemotherapy.
Fig. 3 (See legend on next page.)

ABC transporters regulated by NF-κB and c-Myc:

A

B

rh-cANGPTL4

p-ERK1/2 (Thr202/Tyr204)

ERK1/2 p44/42

β-tubulin

PKBα (Ser473)

PKBβ (Ser473)

p-cMYC (Ser62)

cMYC

pNF-κBp65 (Ser276)

NF-κBp65

β-tubulin

C

D

ABC transporters regulated by NF-κB:

ABC transporters regulated by c-Myc:

ABC transporters regulated by c-Myc:

ABC transporters regulated by c-Myc:

ABC transporters regulated by c-Myc:

ABC transporters regulated by c-Myc:

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Conclusions

We reveal the relationship between EMT-associated metabolic re-wiring and MDR involves metastasis-related gene ANGPTL4, a driver of the EMT-enriched metabolic programme. ANGPTL4 up-regulates multiple ABC transporters expression in cancer cells during EMT via the activation of NF-κB and c-Myc transcription factors. ANGPTL4 deficiency inhibits metabolic flexibility and deters the development of EMT-mediated chemoresistance in metastatic tumor against cisplatin in vivo.

Additional file

Additional file 1: Supplementary Information. (PDF 1910 kb)

Abbreviations

4-OHT: 4-hydroxytamoxifen; ABC: ATP-binding cassette; ANGPTL4: Angiopoietin-like 4; CK18: Cytokeratin 18; Dox: Doxycycline diet; EMT: Epithelial-mesenchymal transition; MDR: Multidrug resistance; qChIP: Quantitative chromatin immunoprecipitation; qPCR: Quantitative real-time PCR; rh-ANPPTL4: Recombinant human ANGPTL4; α-ANGPTL4: Antibody against ANGPTL4; ΔANGPTL4: siRNA knockdown against ANGPTL4

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Availability of data and materials

The datasets for microarray analysis during the current study are available through the Gene Expression Omnibus Series accession number GSE71280. The data in Additional file 1: Figure S6 are in whole based upon data generated by the TCGA Research Network (http://cancergenome.nih.gov/).

Authors’ contributions

NST, MMKL, and PZ designed, performed the experiments and interpreted the data. ZT, DC, ML and YL performed the FACS analysis and chemoresistance analysis. ZT and PZ performed the kinase inhibitor screen and IPA analysis. AWW and MMKL performed the FACS analysis for in vivo animal experiments. JCS performed the quantitative ChIP experiments. PZ and MMKL assisted in data analysis and energy charge experiments. WWBG performed analysis using the TCGA database. NST, MMKL, and PZ wrote the manuscript. NST supervised the entire study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal experiments were carried out in accordance with the guidelines of the institutional animal care and use committee (ARF-SBS/NIE-A0352A, -A0324 and -A0321) of Nanyang Technological University, Singapore.

Consent for publication

All authors have read and approved the final manuscript, and consent to publish.

Competing interests

The authors declare that they have no competing interests.

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References

1. Gillet JP, Gottesman MM. Mechanisms of multidrug resistance in cancer. Methods Mol Biol. 2010;596:47–76.
2. Zheng X, Carstens JL, Kim J, Scheible M, Kaye J, Sugimoto H, Wu CC, LeBlue VS, Kalluri R. Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. Nature. 2015; 527:525–30.
3. Fischer KR, Durrans A, Lee S, Sheng J, Li F, Wong ST, Choi H, El Rayes T, Ryu S, Troeger J, et al. Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. Nature. 2015;527:472–6.
4. Wu CP, Calcagno AM, Ambudkar SV. Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: evaluation of current strategies. Curr Mol Pharmacol. 2008;1:93–105.
5. Tan ML, Teo Z, Sng MK, Zhu P, Tan NS. Emerging roles of angiopoietin-like 4 in human cancer. Mol Cancer Res. 2012;10:677–88.
6. Zhu P, Tan MJ, Huang RL, Tan CK, Chong HC, Pal M, Lam CR, Boukamp P, Pan JY, Tan SH, et al. Angiopoietin-like 4 protein elevates the prosurvival intrinsic O2(•−)/H2O2 ratio and confers anoikis resistance to tumors. Cancer Cell. 2011;19:401–15.
7. Teo Z, Sng MK, Chan JSK, Lim MMK, Li Y, Li L, Phua T, Lee JYH, Tan ZW, Zhu P, Tan NS. Elevation of adenylate energy charge by angiopoietin-like 4 enhances epithelial-mesenchymal transition by inducing 14-3-3gamma expression. Oncogene. 2017;36:6408–19.
8. Tan ZW, Teo Z, Tan C, Choo CC, Loo WS, Song Y, Tan ZY, Ng SP, Koh HZ, Ng YS, et al. ANGPTL4 T266M variant is associated with reduced cancer invasiveness. Biochim Biophys Acta. 2017;1864:1525–36.

9. Porro A, Haber M, Diolaiti D, Iraci N, Henderson M, Gherardi S, Valli E, Munoz MA, Xue C, Flemming C, et al. Direct and coordinate regulation of ATP-binding cassette transporter genes by Myc factors generates specific transcription signatures that significantly affect the chemoresistance phenotype of cancer cells. J Biol Chem. 2010;285:19532–43.

10. Morandi A, Indraccolo S. Linking metabolic reprogramming to therapy resistance in cancer. Biochim Biophys Acta. 2018;1868;1–6.