The effects and possible mechanism of action of apolipoprotein M on the growth of breast cancer cells

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Abstract

Background: To investigate the effects and mechanism of action of apolipoprotein M (ApoM) on the growth of breast cancer (BC) cells.

Methods and Results: Bioinformatics, cell experiments and animal experiments were used to verify the effect of ApoM on breast cancer cell lines and breast tumor growth in vivo. ApoM expression was significantly reduced in BC tissues, and patients with lower ApoM mRNA expression had a poorer prognosis ($P < 0.0001$). Besides, ApoM can partially inhibit the proliferative, migratory and invasive processes of BC cells. In vivo, the difference between ApoM-OE and NC groups was no significant. The level of vitamin D receptor (VDR) protein in MDA-MB-231 cells was increased by overexpression of ApoM ($P < 0.05$), while in MCF-7 cells, VDR levels decreased ($P < 0.05$).

Conclusions: ApoM can partially inhibit the growth of BC cells. VDR may play a role, but is not the main pathway.

Introduction

In 2020, for the first time, breast cancer (BC) topped the list of new cancer cases in the report of global cancer burden. New cases and deaths from BC in Asia accounted for 45.4% and 50.5%, respectively, of the total worldwide[1]. BC is divided into several subtypes based on the presence or absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) receptor: luminal A type (ER+, PR+/-, HER2-), luminal B type (ER+, PR+/-, HER2+), basal-like type/triple negative type (ER-, PR-, HER2-) and HER2+.[2] Among these subtypes, triple-negative BC (TNBC) is the most malignant and accounts for about 15% to 20% of all BC patients.[3] The degree of malignancy of the HER2+ subtype is second only to TNBC, accounting for about 15%–20% of BC. [4] The luminal subtypes that express hormone receptors represent the lowest degree of malignancy, and are present in more than 75% of BC patients.[5] Chemotherapy is the main treatment for TNBC, anti-HER2 therapy is often used for HER2+ BC patients, and endocrine therapy has become the major treatment for luminal BC patients after surgery as it is less toxic and has fewer side effects. Although these treatments have achieved a certain curative effect and addressed some long-term problems faced by clinicians, they have also resulted in new problems, such as increased drug resistance and toxicity toward organs, among others. Therefore, it is still necessary to find new molecules for clinical treatments that can assist existing drug combination therapies or play a role in monitoring their therapeutic effects.[6]

The association of apolipoprotein family members with the occurrence and development of BC has been studied. For example, apolipoprotein D has been found to increase the risk of, affect the development and metastasis of, and be used as a prognostic marker for BC.[7] Apolipoprotein E in tumor tissues plays a role in inhibiting angiogenesis and tumor cell proliferation, [8] but its role in BC is unclear because of its gene polymorphisms.[9] Apolipoprotein C-I helps to distinguish TNBC from non-TNBC and influences the prognosis of patients with TNBC.[10] Another member of the apolipoprotein family, apolipoprotein M

(ApoM), was isolated from chylomicron by Xu et al in 1999.[11] The chemical structure of ApoM is very similar to other members of the apolipoprotein family, as is its role in transporting lipids and regulating lipid metabolism. In addition, our research group has found that ApoM may play a role in the occurrence and development of colorectal cancer, larynx carcinoma and lung cancer, [12-14] but its role in BC is not clear.

Vitamin D receptor (VDR) is a member of the thyroid steroid receptor superfamily. VDR is involved in the regulation of a variety of biological processes, and plays an anti-inflammatory and anti-tumor role in intestine and bone.[15, 16] Huss et al measured the expression of VDR in 718 invasive breast tumors using tissue microarrays, and found that high expression of VDR in invasive breast tumors was associated with good prognosis and low risk of death.¹⁶ Our previous study found that VDR levels significantly increased in colorectal cancer cells when ApoM was overexpressed, and ApoM could inhibit the proliferation and migration of hepatocellular carcinoma cells through the vitamin D receptor signaling pathway.[17, 18] Thus, we aim to explore the effects of ApoM, and its mechanism of action, on the growth of BC cells.

Materials And Methods

With the approval of the Ethics Committee of the Third Affiliated Hospital of Soochow University, the experimental materials and protocols involved in this study were carried out. Informed consent was obtained from, and procedures explained to, all patients in accordance with institutional guidelines and regulations.

Patients and samples

Fifty-five women with BC (age range: 42–83 years) were included in this study. Tumor tissue samples and their corresponding tumor-adjacent tissue samples (not less than 2 cm from the tumor site) were collected during surgery. All samples were quickly frozen in liquid nitrogen after excision and stored at -80°C.

UALCAN and bc-genexminer 4.5 were used to analyse ApoM expression in BC tissue samples. The Kaplan–Meier Plotter database was used to analyse the relationship between ApoM expression and prognosis of BC patients, and the risk ratio, 95% confidence interval (CI) and log-rank P value were used as basic parameters.[19]

Cell culture

The human BC cell lines MCF-7 and MDA-MB-231 were purchased from the Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. MCF-7 was cultured in DMEM (Gibco, Life Technologies, NY, USA) supplemented with 10% foetal bovine serum (FBS) (Gibco, Life Technologies, Melbourne, Australia) and incubated at 37°C with 5% CO₂ in a humidified atmosphere. Leibovitz's L-15 medium (Gibco, Life Technologies, NY, USA) containing 10% FBS was used to culture

MDA-MB-231 cells at 37°C without CO₂. The cells were then digested with trypsin, which was neutralized with complete medium, and the supernatant then discarded by centrifugation. Lastly, the cells were resuspended in fresh complete medium and placed in a suitable medium for further culture.

Lentivirus transfection

Lentivirus carrying the ApoM gene (H14570: pSLenti-SFH-EGFP-P2A- Puro-CMV-ApoM-3Flag, Fig. 2c) and its negative control vector (GL120: pSLenti-SFH-EGFP-P2A-Puro-CMV-MCS-3Flag, Fig. 2b) was synthesized by OBiO Technology (Shanghai, China). BC cells were transfected with lentivirus, and divided into negative control (NC) and ApoM overexpression (ApoM-OE) groups. In order to enhance transfection efficiency, polybrene was added into the cell medium with the lentivirus. After 72 hours of transfection, EGFP-positive cells were observed and photographed under a fluorescence microscope.

Cell proliferation assay

Cell-counting-kit 8 (Dojindo, Kumamoto, Japan) was used to measure cell proliferation. Lentivirus-infected BC cells were plated onto 96-well plates at a density of 1×10^5 cells/mL, with 6 duplicate wells in each group and 100 µl cell suspension in each well. When the cells had adhered to the plate, 10 µL of CCK-8 solution was added to each well and incubated for 2 h, and optical density at 450 nm was used to estimate the number of living cells in each well at 0 h, 24 h, 48 h and 72 h.

Wound healing assay

When the cells were >90% confluent, the culture medium was discarded and several parallel lines were scratched in the cell layer to produce a "wound" on the bottom of the culture plate after washing twice with PBS. The scratch was produced by the tip of a 200 µl sterile pipette oriented perpendicular to the bottom of the culture plate. The cells scraped loose by scratching were decanted with PBS, and 2 mL of medium supplemented with 1% BSA were added after the PBS was discarded. Wounds produced at 0 h, 24 h, 48 h and 72 h was recorded by camera on an inverted microscope, and the percentage of wound healing was recorded.

Cell invasion assay

Before seeding, transwell chambers (Corning, USA) were coated with matrigel (BD Biosciences, San Jose, CA). The cells in the ApoM-OE and NC groups were resuspended in basic culture and seeded into the upper chamber at an appropriate density. The lower chamber was filled with 800 µl complete medium containing 20% FBS. After incubating at 37°C for 48 h, the chambers were soaked in 4% paraformaldehyde for 30 min, followed by staining with crystal violet for 5 min. Six randomly-selected fields of the matrigel were chosen and the number of cells that had invaded were recorded under an inverted microscope.

RNA extraction and reverse transcription

Cells in the logarithmic growth phase were plated at an appropriate density in six-well plates. When the density of cells reached more than 90%, they were lysed and RNA extracted according to the RNA extraction kit (Beyotime, Shanghai, China) instructions. A spectrophotometer was used to measure the absorbance of RNA at 260/280 nm, which reflected the purity and concentration of RNA. A first strand cDNA synthesis kit (Thermo Scientific) was used for reverse transcription of RNA.

Quantitative real-time PCR

Gene expression was detected by the Light Cycler 480II PCR system. The total volume of the amplification system was 25 μ L, containing 2.5 μ L 10 $\times$ buffer, 0.5 μ L 10 mM dNTPs, 2.5 μ L 25 mM MgCl₂, 0.4 μ L 10 μ M primers and probes, 0.25 μ L DNA polymerase, and 2 μ L sample template. The thermal cycling conditions included initial denaturation at 95°C for 3 min, 5 sec at 95°C and 15 sec at 60°C for 40 cycles. The relative expression of target genes was quantified using $2^{-\Delta C_t}$. [20] The sequences of primers and probes are provided in Table 1.

In vivo tumor xenograft

To establish stable breast tumors in situ, an estrogen-releasing pellet (Innovative Research, America) was implanted into the backs of mice and MCF-7 cells were injected into mammary fat pads of BALBC mice. Tumor volume was calculated as $0.5 \times \text{length} \times \text{width}^2$ and recorded twice weekly. When tumor volume reached 90 mm³, 10 μ L of adeno-associated virus (AAV) overexpressing ApoM and its negative control AAV was injected into the tumor at each of several sites. Tumor volume and body weight were continuously measured and recorded. When mice were sacrificed on the 30th day, tumors were removed AV, weighed and photographed. The tumor tissues were then divided into two parts, one for measurement of ApoM over-expression and the other for frozen sections.

Western blotting

BC cells were washed by ice-cold PBS and lysed using a total protein extraction kit (Bestbio, China). Protein was quantified by a BCA protein quantitative kit (Bestbio, China). Loading buffer was used to mix with protein samples. After boiling for 10 min, the protein was denatured. Protein was separated under 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA, USA). 5% skimmed milk powder was used to block the membrane at room temperature, and anti-ApoM (GeneTex 83083, USA) or anti-VDR (ab109234, Abcam) antibodies were incubated with the membrane at 4 °C overnight. After washing several times with tris buffered saline with tween, membranes were incubated with horseradish peroxidase-conjugated affinipure anti-mouse IgG (SA00001-1, Proteintech) or anti-rabbit IgG (SA00001-2, Proteintech). ECL Western Blotting Substrate kit (Thermo Scientific, Rockford, IL, USA) was utilized to visualize protein bands using an automatic chemiluminescence image analysis system. β -actin was used to normalize protein loading.

Statistical analysis

GraphPad Prism 8.0 software (Inc, San Diego, CA) was used for statistical analysis. The data are presented by mean $\pm$ standard deviation. Wilcoxon matched-pairs signed rank tests, one-way ANOVA and student's t-tests were used to compare differences among groups. A *P* value less than 0.05 was considered statistically significant.

Results

Expression of ApoM in breast tumor tissues and its relationship with prognosis.

ApoM expression was significantly different between BC (n=1097) and normal tissues (n=114, $P<0.0001$, Fig. 1a), and ApoM levels were significantly lower in all subtypes of BC compared with normal subjects ($P<0.0001$, Fig. 1c). In tumor tissues from 55 BC patients, ApoM mRNA levels were significantly lower than in adjacent tissues ($P=0.0345$, Fig. 1e). Patients were divided into high (>0.006524) and low (<0.006524) ApoM mRNA expression groups, between which there was no significant difference in clinicopathological indicators ($P>0.05$, Table 2).

There was follow up of fifty-five patients for 40 months, of which 9 cases were lost, 1 case developed cardiac cancer and right supraclavicular lymph node metastasis at the 24th week after operation, and the rest of the patients had no recurrence. Among 3458 lymph node positive (N+) and 4431 lymph node negative (N-) breast cancer patients, ApoM expression was significantly lower in the N+ patients than in the N- patients ($P<0.0001$, Fig. 1b). Survival analysis of 4929 BC patients showed that those with lower ApoM mRNA expression tended to have a poorer prognosis ($P<0.0001$, Fig. 1d). The level of ApoM had some value in predicting the outcomes of normal vs. BC patients (AUC=0.673, CI=0.634-0.712).

The effect of ApoM on the proliferation, migration and invasive ability of BC cells.

RT-PCR results showed that the relative expression of ApoM mRNA in MCF-7, ZR-75-1, BT-474, MDA-MB-231 and MDA-MB-468 cells was low, less than 1×10^{-3} (Fig. 2a). Experiments were carried out on ApoM-OE MCF-7 and MDA-MB-231 cells. In CCK-8 assays, cell proliferation of ApoM-OE groups was significantly lower than that of NC groups (Fig. 2f); the reduction for MCF-7 cells was especially notable from the 24th hour ($P \leq 0.0001$). In wound-healing experiments (Fig. 2g), the healing ability of the ApoM-OE group was significantly less than that of the NC group in MDA-MB-231 cells ($P \leq 0.0001$). In transwell assays, the invasive ability of MCF-7 cells in the ApoM-OE group was less than that in the NC group, $P \leq 0.001$.

The effect of ApoM on the growth of mouse BC tumors.

The results of RT-PCR showed that the level of ApoM mRNA in the ApoM-OE group was 2.161 times that in the NC group (Fig. 3b,d). Within 30 days, the average body weight in the NC group had decreased by 0.44 g, while the body weight in the ApoM-OE group had increased by 1.19 g. There was no significant difference in body weight between the two groups (Fig. 3c). The tumor masses of the NC and ApoM-OE groups were 0.9393 ± 0.7561 g and 0.7221 ± 0.3316 g, respectively, although the difference was not

statistically significant (Fig. 3f). In addition, there was no correlation between ApoM mRNA and tumor mass (Fig. 3g).

ApoM may regulate VDR expression and affect BC progression.

In MDA-MB-231 cells, overexpression of ApoM inhibited VDR expression at both the mRNA and protein levels, with $P=0.0243$ and $P=0.0179$, respectively (Fig. 4b). In contrast, overexpression of ApoM up-regulated the levels of VDR protein in MCF-7 cells ($P=0.0157$, Fig. 4a).

Discussion

ApoM is a transport carrier of lipoproteins, mainly exists in HDL particles, and plays a key role in the maturation of HDL. As a secreted protein, ApoM is selectively and highly expressed in liver cells and renal tubular epithelium, and participates in lipid metabolism and maintenance of kidney function. ApoM is the main carrier of sphingosine-1-phosphate (S1P). In recent years, studies have found that the ApoM-S1P axis can not only inhibit HUVEC inflammation through the PI3K/Akt signaling pathway, [21] but also promote repair of damage to animal lung and kidney function.[22, 23] In oncology research, our team not only found that ApoM expression was significantly reduced in colorectal cancer, but also discovered that it could promote progression of non-small cell lung cancer by up-regulating the expression of S1P receptors. ApoM has been demonstrated to inhibit proliferation and migration of hepatocarcinoma cells via the VDR signaling pathway.[17] Some studies have reported that VDR can reduce BC risk, and that VDR and vitamin D can regulate autophagy and death of BC cells. In addition, S1P can inhibit motility and proliferation in BC cells.[24] The question then arises as to whether ApoM, which is an S1P carrier, participates in the occurrence and development of BC. At present, there are few reports on the role of ApoM in the development of BC, and this study carried out a preliminary exploration of this subject.

We found that expression of ApoM in tumor tissues was significantly lower than in normal tissues, and was closely related to the prognosis of BC patients using bioinformatics analysis. We selected ER+ (MCF-7) and ER- (MDA-MB-231) BC cell lines for cell experiments. The results showed that ApoM inhibited the proliferation, migration and invasion of the two cell lines.

In experiments in vivo, however, the number of experimental animals in each group was too small and the individuals too variable to yield statistically significant differences. There are many kinds of AAV serotypes that target different organs, [25] but at present there is no serotype targeting the mammary gland. In order to target the virus to tumors with high efficiency, the AAV2/9 hybrid serotype virus was injected into tumors by multi-point in situ injection. However, the body weight, tumor volume and tumor mass were not significantly different between the NC and ApoM-OE groups, which indicated that ApoM had no effect on the growth of breast tumors in vivo. In the experiments, the fluorescence intensity and uniformity of EGFP carried by tumor cells in the NC and ApoM-OE groups were low, while the level of ApoM mRNA in the ApoM-OE group was only 2.161 times higher than that in the NC group, which indicates that in situ injection of AAV2/9 did not achieve high transfection and ApoM over-expression efficiency. Furthermore, the current experimental design mainly verified that ApoM had little therapeutic

effect on breast tumors. In the future, we expect that expanding the sample size, improving the tissue specificity of the AAV serotype, changing the AAV injection method, and using in vivo imaging to observe the expression of virus will more clearly show an effect of ApoM on breast tumors.

VDR is a transcription factor that regulates various biological processes, including proliferation, differentiation, apoptosis, and angiogenesis, among others.[26] The expression of VDR and GAPDH in colorectal cancer HT-29 cells has been detected by single-tube double RT-qPCR, and it was found that ApoM could up-regulate expression of VDR mRNA.[27] In order to detect whether VDR expression in BC cell lines is affected by ApoM overexpression, we used PCR and western blotting. The results showed that an increase in ApoM expression resulted in less VDR expression in ER+ MCF-7, but more in ER- MDA-MB-231 BC cells. It has been reported that estrogen can down-regulate VDR expression in ER+ BC cell lines. [16] Therefore, we speculate that VDR expression in BC cell lines is not only related to the level of ApoM, but also affected by estrogen, and in ER+ BC cells, the regulatory effect of estrogen on VDR may be greater than that of ApoM. In follow-up experiments we need to further explore the regulatory mechanism of ApoM on VDR expression in BC cells.

Conclusion

ApoM can partially inhibit the growth of BC cells. VDR may be involved in this process, but it is not the main pathway. The effect of ApoM on the growth of breast tumors in vivo is as yet unclear, and further study is needed.

Declarations

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Conflicts of interest: The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material: Not applicable.

Code availability: Not applicable.

Authors' contributions: Guanghai Luo, Ning Xu and Miaomei Yu contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ying Zhou and Shuang Yao. The first draft of the manuscript was written by Ying Zhou and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval: The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of the third affiliated hospital of Soochow University.

Consent to participate: Participants provided written informed consent before enrolment.

Consent for publication (include appropriate statements): Not applicable.

References

1. Cancer IAfro Cancer Today
2. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lunning PE, Borresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 98:10869–10874. doi:10.1073/pnas.191367098
3. Yin L, Duan JJ, Bian XW, Yu SC (2020) Triple-negative breast cancer molecular subtyping and treatment progress. *Breast Cancer Res* 22:61. doi:10.1186/s13058-020-01296-5
4. Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JMS, Bilous M, Ellis IO, Fitzgibbons P, Hanna W, Jenkins RB, Press MF, Spears PA, Vance GH, Viale G, McShane LM, Dowsett M (2018) Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. *J Clin Oncol* 36:2105–2122. doi:10.1200/jco.2018.77.8738
5. Panet F, Couture F, Kwiatkowska A, Desjardins R, Guerin B, Day R (2017) PACE4 is an important driver of ZR-75-1 estrogen receptor-positive breast cancer proliferation and tumor progression. *Eur J Cell Biol* 96:469–475. doi:10.1016/j.ejcb.2017.03.006
6. Loibl S, Gianni L (2017) HER2-positive breast cancer. *Lancet* 389:2415–2429. doi:10.1016/S0140-6736(16)32417-5
7. Lea OA, Kvinnsland S, Thorsen T (1987) Progesterone-binding cyst protein in human breast tumor cytosol. *Cancer Res* 47:6189–6192
8. Zhou Y, Luo G (2020) Apolipoproteins, as the carrier proteins for lipids, are involved in the development of breast cancer. *Clin Transl Oncol*. doi:10.1007/s12094-020-02354-2
9. Liu Y-L, Zhang H-M, Pan H-M, Bao Y-H, Xue J, Wang T-C, Dong X-C, Li X-L, Bao H-G (2016) The relationship between apolipoprotein E gene epsilon 2/epsilon 3/epsilon 4 polymorphism and breast cancer risk: a systematic review and meta-analysis. *Oncotargets Therapy* 9:1241–1249. doi:10.2147/ott.s94228
10. Song D, Yue L, Zhang J, Ma S, Zhao W, Guo F, Fan Y, Yang H, Liu Q, Zhang D, Xia Z, Qin P, Jia J, Yue M, Yu J, Zheng S, Yang F, Wang J (2016) Diagnostic and prognostic significance of serum apolipoprotein C-I in triple-negative breast cancer based on mass spectrometry. *Cancer Biol Ther* 17:635–647. doi:10.1080/15384047.2016.1156262
11. Xu N, Dahlback B (1999) A novel human apolipoprotein (apoM). *J Biol Chem* 274:31286–31290
12. Zhu Y, Luo G, Jiang B, Yu M, Feng Y, Wang M, Xu N, Zhang X (2018) Apolipoprotein M promotes proliferation and invasion in non-small cell lung cancers via upregulating S1PR1 and activating the

- ERK1/2 and PI3K/AKT signaling pathways. *Biochem Biophys Res Commun* 501:520–526. doi:10.1016/j.bbrc.2018.05.029
13. Mu QF, Luo GH, Chen LJ, Wei J, Zheng L, Zhang XY, Xu N (2012) [Apolipoprotein M expression in human colorectal cancer tissues and its clinicopathological relevance]. *Zhonghua Wei Chang Wai Ke Za Zhi* 15:855–858
 14. Xue H, Yu M, Zhou Y, Zhang J, Mu Q, Chen T, Luo G, Liu J (2020) Apolipoprotein M inhibits proliferation and migration of larynx carcinoma cells. *Sci Rep* 10:19424. doi:10.1038/s41598-020-76480-w
 15. Bakke D, Sun J (2018) Ancient Nuclear Receptor VDR With New Functions: Microbiome and Inflammation. *Inflamm Bowel Dis* 24:1149–1154. doi:10.1093/ibd/izy092
 16. Huss L, Butt ST, Borgquist S, Elebro K, Sandsveden M, Rosendahl A, Manjer J (2019) Vitamin D receptor expression in invasive breast tumors and breast cancer survival. *Breast Cancer Res* 21:84. doi:10.1186/s13058-019-1169-1
 17. Yu M, Pan L, Sang C, Mu Q, Zheng L, Luo G, Xu N (2019) Apolipoprotein M could inhibit growth and metastasis of SMMC7721 cells via vitamin D receptor signaling. *Cancer Manag Res* 11:3691–3701. doi:10.2147/CMAR.S202799
 18. Yu M-M, Yao S, Luo K-M, Mu Q-F, Yu Y, Luo G-H, Xu N (2017) Apolipoprotein M increases the expression of vitamin D receptor mRNA in colorectal cancer cells detected with duplex fluorescence reverse transcription-quantitative polymerase chain reaction. *Mol Med Rep* 16:1167–1172. doi:10.3892/mmr.2017.6716
 19. Györfy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, Szallasi Z (2010) An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res Treat* 123:725–731. doi:10.1007/s10549-009-0674-9
 20. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3:1101–1108. doi:10.1038/nprot.2008.73
 21. Zheng Z, Zeng Y, Zhu X, Tan Y, Li Y, Li Q, Yi G (2019) ApoM-S1P Modulates Ox-LDL-Induced Inflammation Through the PI3K/Akt Signaling Pathway in HUVECs. *Inflammation* 42:606–617. doi:10.1007/s10753-018-0918-0
 22. Ding BS, Yang D, Swendeman SL, Christoffersen C, Nielsen LB, Friedman SL, Powell CA, Hla T, Cao Z (2020) Aging Suppresses Sphingosine-1-Phosphate Chaperone ApoM in Circulation Resulting in Maladaptive Organ Repair. *Dev Cell* 53:677–690.e4. doi:10.1016/j.devcel.2020.05.024
 23. Hajny S, Christoffersen C (2017) A Novel Perspective on the ApoM-S1P Axis, Highlighting the Metabolism of ApoM and Its Role in Liver Fibrosis and Neuroinflammation. *Int J Mol Sci* 18. doi:10.3390/ijms18081636
 24. Arkensteijn BW, Berbee JF, Rensen PC, Nielsen LB, Christoffersen C (2013) The apolipoprotein m-sphingosine-1-phosphate axis: biological relevance in lipoprotein metabolism, lipid disorders and atherosclerosis. *Int J Mol Sci* 14:4419–4431. doi:10.3390/ijms14034419

25. Aponte-Ubillus JJ, Barajas D, Peltier J, Bardliving C, Shamlou P, Gold D (2018) Molecular design for recombinant adeno-associated virus (rAAV) vector production. *Appl Microbiol Biotechnol* 102:1045–1054. doi:10.1007/s00253-017-8670-1
26. Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ (2014) The role of vitamin D in reducing cancer risk and progression. *Nat Rev Cancer* 14:342–357. doi:10.1038/nrc3691
27. Yu MM, Yao S, Luo KM, Mu QF, Yu Y, Luo GH, Xu N (2017) Apolipoprotein M increases the expression of vitamin D receptor mRNA in colorectal cancer cells detected with duplex fluorescence reverse transcription-quantitative polymerase chain reaction. *Mol Med Rep* 16:1167–1172. doi:10.3892/mmr.2017.6716

Tables

Table 1
Sequences of the primers and probes used in this study

Gene	Primer/Probe	Sequences(5'→3')
GAPDH	Forward primer	CAGGGCTGCTTTTAACTCTGGT
	Reverse primer	CATGGGTGGAATCATATTGGAAC
	Probe	Cy5-TGGATATTGTTGCCATCAATGACCCCT-BHQ2
APOM	Forward primer	CTGACAACCTCTGGGCGTGGAT
	Reverse primer	TGTCCACAGGGTCAAAAGTTGC
	Probe	FAM-AGTTCCCAGAGGTCCACTTGGGCCA-BHQ1
VDR	Forward primer	GCTAAGATGATACCAGGATTCAGAGAC
	Reverse primer	AAGGACTCATTGGAGCGCAAC
	Probe	FAM-ACCTCTGAGGACCAGATCGTACTGCTGA-BHQ1

Table 2
Correlation between ApoM mRNA levels and clinicopathological indicators

Characteristic	ApoM mRNA level		χ^2	P value
	high	low		
Age			0.9391	0.3325
< 55	14	10		
≥ 55	14	17		
Tumor			0.01408	0.9055
T1	12	12		
T2 ~ T4	16	15		
Lymph node metastasis			1.467	0.2258
Negative	19	14		
Positive	9	13		
ER			2.194	0.1385
Negative	17	11		
Positive	11	16		
PR			2.195	0.1385
Negative	19	13		
Positive	9	14		
Ki-67 (%)			1.039	0.3080
≤ 20	12	8		
> 20	16	19		

Figures

Figure 1

The expression of ApoM in BC tissues and its relationship to prognosis. (a) ApoM expression in tumors was significantly lower than in normal tissues, $P < 0.0001$; (b) ApoM expression was significantly lower in N+ patients than in N- patients, $P < 0.0001$; (c) ApoM levels were significantly lower in all subtypes of BC compared with normal subjects, $P < 0.0001$; (d) survival analysis showed that patients with lower ApoM mRNA expression tended to have a poorer prognosis, $P < 0.0001$; (e) ApoM mRNA levels in 55 BC patients' tumor tissues were significantly lower than in adjacent tissues, $P = 0.0345$; (f) ApoM was partially predictive of outcomes in normal and cancer patients (AUC=0.673, CI=0.634-0.712).

Figure 2

The effects of ApoM on the biological behavior of BC cell lines. (a) Expression of ApoM was low in five BC cell lines; (b) GL120: pSLenti-SFH-EGFP-P2A-Puro-CMV-MCS-3Flag; (c) H14570:pSLenti-SFH-EGFP-P2A-Puro-CMV-ApoM-3Flag; (d) transfection of ApoM in MCF-7 and MDA-MB-231 cell lines under fluorescence microscopy; (e) overexpression efficiency of ApoM in BC cells; (f) ApoM inhibits proliferation of MCF-7 cells; (g) ApoM inhibits migration of MDA-MB-231 cells; (h) ApoM inhibits the invasion by MCF-7 cells.

Figure 3

The effects of ApoM on breast tumors in vivo. (a) Empty vector AOV024:pAAV-CMV-EGFP-P2A-3FLAG-WPRE, vector H17612 carrying ApoM:pAAV-CMV-EGFP-P2A-APOM-3FLAG-WPRE; (b) transfection of ApoM in breast tumors under fluorescence microscopy; (c) ApoM had no effect on body weight and tumor volume; (d) overexpression of ApoM in breast tumors; (e) in vivo and in vitro examples of mouse tumors; (f) the difference between NC and ApoM-OE groups was not significant; (g) ApoM had no correlation with tumor mass in 12 animals.

Figure 4

The effects of ApoM-overexpression on VDR levels in BC cell lines. (a) ApoM up-regulated the levels of VDR protein in MCF-7 cells, $P=0.0157$; (b) ApoM inhibited the expression of VDR in MDA-MB-231 cells.