Chinese-German J Clin Oncol
DOI 10.1007/s10330-014-1328-x
June 2014, Vol. 13, No. 6, P254–P258

Relationship between epithelial to mesenchymal transition and chemoresistance of lung cancer

Yunqing Chen, Jin Wang (Co-first author), Fenggang Xiang, Min Li

Department of Pathology, Qingdao University, Qingdao 266000, China

Received: 8 May 2014 / Revised: 22 May 2014 / Accepted: 5 June 2014

Abstract ***Objective:*** The aim of this study was to explore the correlation between epithelial to mesenchymal transition (EMT) and chemoresistance of non-small-cell lung cancer (NSCLC). ***Methods:*** *In vitro*, the drug resistance index of cisplatin resistant lung adenocarcinoma cell line (A549/DDP) was detected by CCK-8 assay; the morphological change between A549/DDP cells and lung adenocarcinoma cells (A549) was observed by phase contrast microscope; expression of EMT markers (including E-cadherin and vimentin) and resistance protein, excision repair cross-complementing 1 (ERCC1) was detected by immunocytochemistry. The expression of E-cadherin, vimentin and ERCC1 was investigated by immunohistochemistry in 120 cases of NSCLC, half of that were treated with pre-operative neoadjuvant chemotherapy (neoadjuvant chemotherapy group), and the other underwent surgery alone (simple surgery group). ***Results:*** There was a significant difference between the IC_{50} (half maximal inhibitory concentration) of A549/DDP cells (5.20) and A549 cells (1.88) ($P < 0.05$), and the drug resistance index of A549/DDP cells was 2.77. Compared with A549 cells, A549/DDP cells increased expression of ERCC1 ($P < 0.05$). Moreover, A549/DDP cells showed morphological and phenotypic changes consistent with EMT: with spindle-shaped morphology, and decreased expression of E-cadherin and increased expression of vimentin. Immunohistochemistry showed significant positive correlation between the expression of ERCCl and vimentin ($r = 0.496$, 0.332, $P < 0.05$), and significant negative correlation between the ERCCl and E-cadherin ($r = –0.403$, –0.295, $P < 0.05$) in neoadjuvant chemotherapy group and simple surgery group. In addition, compared with simple surgery group, the expression of ERCC1 ($P = 0.003$) and vimentin (P = 0.004) was significantly increased, and the expression of E-cadherin was decreased in neoadjuvant chemotherapy group ($P = 0.032$). ***Conclusion:*** A549/DDP cells acquired cisplatin-resistance and occurred EMT simultaneously; the phenomenon of chemoresistance and EMT was caused more easily in neoadjuvant chemotherapy group. As such, we further confirmed the close correlation between chemoresistance and EMT of NSCLC, and provided theoretical basis for the targeting therapy with EMT regulatory factor for chemoresistant NSCLC patients.

Key words epithelial to mesenchymal transition; chemoresistance; lung caner

Worldwide, lung cancer was the most commonly diagnosed and most fatal cancers in males, while having the fourth highest incidence rate and second highest mortality rate in females. The total 5-year survival rate is less than 15% [1]. Surgery is considered the best treatment for non-small-cell lung cancer (NSCLC). However, lung cancer is usually found at an advanced stage, and fewer than 25% of NSCLC patients are considered fit for surgery [2]. Chemotherapy is the main adjuvant therapeutic strategy for cancer treatment, but the chemoresistance often leads to the failure of chemotherapy [3].

Epithelial to mesenchymal transition (EMT) refers to the process that epithelial cells change to mesenchymal cells, during which epithelial cells lose their polarity and acquired increased motility and invasion [4–7]. EMT has been shown to be essential for embryonic development, neural nest and development of heart and other tissues and organs [4]. Recent studies have extended the knowledge that EMT is also involved in organ fibrosis, tissue repair, and cancer progression [5]. Accumulating studies demonstrated that EMT is involved in the metastasis, treatment resistance and associated with the progression of many type of tumors [6–7].

In order to explore the correlation between chemoresistance and EMT, we observed the changes of morphology and expression of EMT markers as well as resistance protein between A549/DDP cells and A549 cells, and the differential expression of resistance protein and EMT marker in neoadjuvant chemotherapy group and simple surgery group.

Correspondence to: Fenggang Xiang. Email: xiangfenggang@163.com

Materials and methods

Cell lines and cell culture

The A549/DDP cells and A549 cells were purchased from the cell bank of Xiangya Central Laboratory. The cell lines were cultured in RPMI-1640 (Hyclone, USA) supplemented with 10% FBS (Zhejiang Tianhang Biological Technology Co., Ltd, China) and 1% penicillin-streptomycin at 37 ˚C in 5% CO_2. Cisplatin (2 μg/mL) was regularly added to A549/DDP cells to maintain their drug resistance.

Drug cytotoxicity assay

The drug resistance index of A549/DDP cells was evaluated by CCK-8 assay (Boster, China). 5×10^3 cells per well were seeded in 96-well plates in 100 μL medium with cisplatin of various concentration (0, 1, 2, 4, 8, 16 μg/mL) and left to incubate for 48 h at 37℃ and 5% CO_2. After 48 h, 10 μL CCK-8 was added to each well and cells were left for 2 h. The absorbance of each well was measured at 450 nm. Each experiment was performed using 5 replicate wells and 3 independent experiments. The dose-response curve could be plotted with the inhibition rate. The IC_{50} (half maximal inhibitory concentration) was defined as the drug concentration required to produce a 50% reduction of optimal density in each test. Resistance index was calculated as IC_{50} resistant/IC_{50} sensitive cell line.

Primary tumor specimens

One hundred and twenty tumor specimens of NSCLC patients were taken from the Affiliated Hospital of Qingdao University Medical College, 60 of which underwent surgery following neoadjuvant chemotherapy in the last 5 years (neoadjuvant chemotherapy group). Each patient received 2 cycles of cisplatin-based chemotherapy. Another group of 60 patients underwent surgery alone without neoadjuvant chemotherapy (simple surgery group) was collected between May 2011 and June 2013. NSCLC was definitively identified based on Pathological Department of the Affiliated Hospital of Qingdao University Medical College in every patient of both groups.

Specimens of tumor were fixed in neutral formalin, processed with graded ethanol, embedded in paraffin, and continuously sectioned (4 μm).

Immunocytochemistry and immunohistochemistry

The slides of cells were fixed in –20 ℃ acetone for 20 min and treated by TritonX-100 for 20 min. The cell slides and specimens were stained by immunocytochemistry and immunohistochemistry according to the product specification (PV6000, ZSGB-BIO). The positive site and dilution of primary antibody used were as follows: ERCC1 (nucleus, 1:100), E-cadherin (membrane/cytoplasm, 1:200) and vimentin (membrane/cytoplasm, 1:100). PBS instead of the primary antibody was used as negative controls. Three slices per cell line and per detection index were divided into 12 visual fields average, using for statistical analysis.

Statistical analysis

The data was statistically analyzed with SPSS19.0 software. The diversity between immune-markers expression was analyzed with the *t* test. The correlation between two variances was analyzed with Spearman rank correlation analysis. *P* values < 0.05 were considered statistically significant.

Results

Verification of drug resistance of A549/DDP cells

The IC_{50} (half maximal inhibitory concentration) of A549/DDP cells and A549 cells was detected by CCK-8 assay to define the drug resistance index of A549/DDP cells. Fig. 1A showed that the cell inhibition rate increased with the increasing concentration of cisplatin. The IC_{50} of A549/DDP cells was significantly higher than that of A549 cells ($P < 0.05$), and the drug resistance index of A549/DDP cells was 2.77 (Table 1). In addition, immunocytochemistry showed that the expression of ERCC1 of A549/DDP was significantly increased ($P < 0.05$) (Table 2, Fig. 1B).

Effects of cisplatin resistance on EMT phenotype of A549 cells

Morphology changes were observed by phase contrast microscope that A549/DDP cells showed a spindle-shaped morphology, while the A549 cells showed polygon shape (Fig. 2A). Then we investigated the protein expression of EMT marker by immunocytochemistry to further explore whether A549/DDP cells occurred EMT. Immunocytchemical results showed that, the expression of E-cadherin was significantly decreased and the expression of vimentin was increased ($P < 0.05$) compared with A549 cells (Table 2, Fig. 2B).

Correlation between the expression of drug resistance protein and EMT maker protein in specimens of NSCLC

Immunohistochemical findings showed that the expression of ERCC1, vimentin and E-cadherin were 76.67% (46/60), 66.67% (40/60) and 28.33% (17/60) in neoadjuvant chemotherapy group, and 55.0% (33/60), 51.67% (31/60) and 31.67% (19/60) in simple surgery group (Table 3). A significant positive correlation between ERCC1 and vimentin in neoadjuvant chemotherapy group ($r = 0.496$, $P = 0.001$) and simple surgery group ($r = 0.332$, $P = 0.06$),

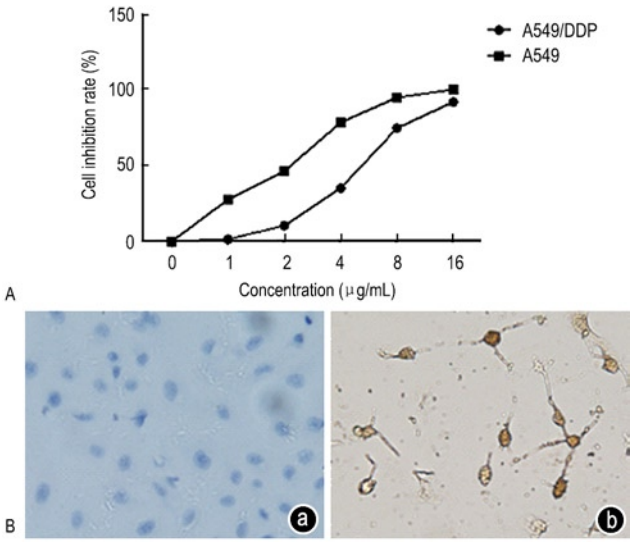

Fig. 1 Verification of drug resistance of A549/DDP cells. (A) A549/DDP and A549 cells were treated with various concentration of cisplatin (0, 1, 2, 4, 8, 16 μg/mL), and the inhibition rate of the two cell lines was detected after 48 h. (B) Immunocytochemistry showed that, compare with A549 cells, the expression of ERCC1 of A549/DDP cells was significantly increased (PV6000 × 200). (a) Expression of ERCC1 (A549); (b) Expression of ERCC1 (A549/DDP)

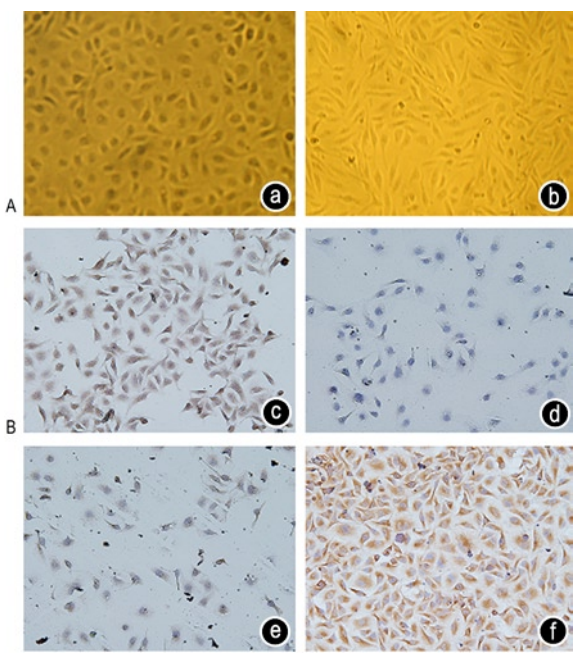

Fig. 2 Morphology characteristics and expression of EMT marker of A549 cells and A549/DDP cells. (A) Observation with phase contrast microscope showed a spindle-shape of A549/DDP cells and a polygon-shape of A549 cells (PV6000 × 200). (B) Immunocytochemistry showed that, compared with A549 cells, the expression of E-cadherin of A549/DDP cells was decreased and the expression of vimentin was increased (PV6000 × 200). (a) A549 cells; (b) A549/DDP cells; (c) Expression of E-cadherin (A549); (d) Expression of E-cadherin (A549/DDP); (e) Expression of vimentin (A549); (f) Expression of vimentin (A549/DDP)

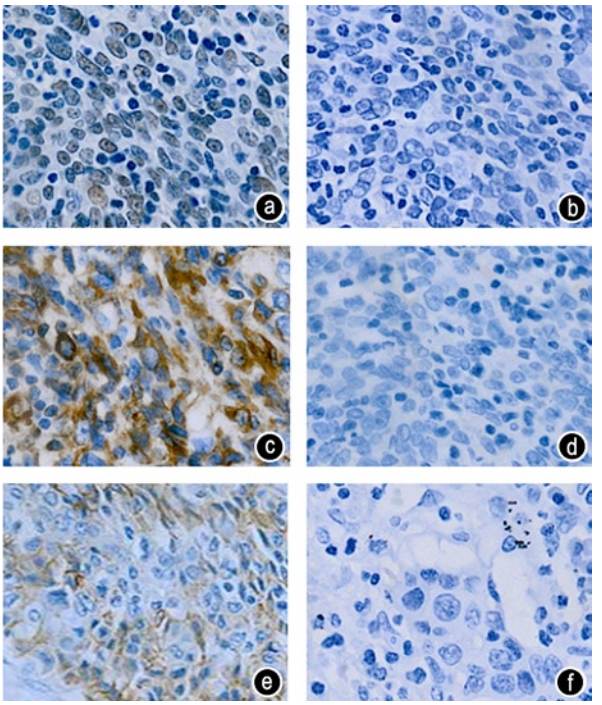

Fig. 3 Immunohistochemical staining results (PV6000 × 200). (a) Positive expression of ERCC1; (b) Negative expression of ERCC1; (c) Positive expression of vimentin; (d) Negative expression of vimentin; (e) Positive expression of E-cadherin; (f) Negative expression of E-cadherin

Table 1 IC_{50} of the two cell lines evaluated by CCK-8 assay

Cell lines	IC_{50} (μg/mL)	Drug resistance index
A549	1.88	–
A549/DDP	5.20	2.77

Table 2 The protein expression of ERCC1, vimentin and E-cadherin of the two cell lines (n = 12)

Cell lines	ERCC1		Vimentin		E-cadherin	
	+	–	+	–	+	–
A549	1	11	7	5	10	2
A549/DDP	9	3	12	0	4	8

Three slices per cell line and per detection index were divided into 12 visual fields average, using for statistical analysis

Table 3 Protein expression of ERCC1, vimentin and E-cadherin (n = 60)

Groups	ERCC1		Vimentin		E-cadherin	
	+	–	+	–	+	–
Neoadjuvant chemotherapy	46	14	40	20	17	43
Simple surgery	33	27	31	29	19	41

and a significant negative correlation between ERCC1 and E-cadherin in neoadjuvant chemotherapy group (r = –0.403, P = 0.004) and simple surgery group (r = –0.295, P = 0.037) (Table 4) were found by Spearman analysis.

Meanwhile, the expression of ERCC1 (P = 0.003) and

Table 4 Correlation between vimentin, E-cadherin and ERCC1 (n = 60)

Groups	ERCC1	Vimentin		E-cadherin	
		+	–	+	–
Neoadjuvant chemotherapy	+	34	12	9	33
	–	6	8	8	10
Simple surgery	+	23	10	13	20
	–	8	19	6	21

vimentin (P = 0.004) was significantly increased, and the expression of E-cadherin was decreased (P = 0.032) compared with simple surgery group.

Discussion

Chemotherapy is the main adjuvant therapeutic strategy for NSCLC treatment [8]. The combined chemotherapy based on cisplatin prolonged the survival time of patients with NSCLC effectively, but their 5-year survival rate was not significantly increased, the main reason is the drug resistance to cisplatin of NSCLC cells [3].

EMT is a fundamental biological process during which epithelial cells lose their polarity and change to a mesenchymal phenotype accompanied by increased motility and invasion [4–7]. Recent studies found the process of EMT widely involved in the acquisition of chemoresistance. Sarkar *et al* found an upregulation of E-cadherin in pancreatic cancer cells sensitive to gefitinib (L3/6pl, Clol357, BxPC-3 and HPAC), while an upregulation of vimentin and ZEB-1 in the pancreatic cancer cells resistant to gefitinib (MiaPaCa-2, Panc-1 and Aspc-1) [9]. Foroni *et al* established breast cancer cell lines resistant to taxol, doxorubicin and docetaxel, and found an increased expression of vimentin and a decreased expression of E-cadherin [10]. In this study, we observed that A549/DDP cells acquired cisplatin-resistance and occurred EMT simultaneously, including a change of morphology that A549/DDP cells showed a spindle-shaped morphology, and phenotypic changes-downregulation of E-cadherin and upregulation of vimentin. We further supplemented the above results from the perspective of EMT and confirmed the correlation between chemoresistance and EMT.

We also showed the positive correlation between vimentin and ERCC1, and the negative correlation between E-cadherin and ERCC1 in neoadjuvant chemotherapy group and simple surgery group. In addition, we found the phenomena of EMT and chemoresistance were more apparent in neoadjuvant chemotherapy group, it indicated that the chemoresistance and EMT were caused more easily in neoadjuvant chemotherapy group, which contributed to the controversy about the curative effect of neoadjuvant chemotherapy treatment [11]. The results above confirmed the correlation between chemoresistance and EMT of NSCLC from the histological level, and consisted with the results of our cell culture experiments.

Our study confirmed the relationship between chemoresistance and EMT from the cytological and histological level, but the underlying mechanism was not clear. Currently, most scholars accepted the mechanism that EMT and chemoresistance have focal points in their signal pathways. The activation and disorder of EMT-related signal transduction pathway regulate both EMT and chemoresistance progress. The EMT signal pathways, including P13K/AKT/GSK-3β/Snail, NF-κB/YY1/RKIP/PTEN, P13K/AKT/HIF-1α, Notch-2 etc, were confirmed to play a key role in the process of drug resistance to cisplatin, gemcitabine, gefitinib etc [12–16]. Moreover, the production of multi-drug resistance (MDR) of tumor cells has also been shown to be accompanied with EMT. Han *et al* found that the activation of P13K/AKT, MAPK signal upregulated the expression of P-gp (the critical transcription regulator of MDR), while the P13K/AKT and MAPK are important pathways of EMT [17]. Furthermore, it was demonstrated that the P-gp protein and ATP-binding cassette (ABC) which were essential for MDR, were mediated by the EMT transcription factor-Snail and Twist [18–20]. The above study results indicated that EMT and MDR exist meeting points in their signal pathways.

The interrelationship between chemoresistance and EMT was extremely complex, and there may be more common regulatory factors or signal pathways. This study provided theoretical basis for the further research about the correlation mechanisms between chemoresistance and EMT. Exploration on the correlation between EMT related signal pathways and chemoresistance will be the focus of future research, and will provide new strategy for treatment on restoring chemotherapy sensitivity for tumor patients.

References

1. Wangari-Talbot J, Hopper-Borge E. Drug resistance mechanisms in non-small cell lung carcinoma. J Can Res Updates, 2013, 2: 265–282.
2. Xiao D, He J. Epithelial mesenchymal transition and lung cancer. J Thorac Dis, 2010, 2: 154–159.
3. Olaussen KA, Dunant A, Fouret P, *et al*. DNA repair by ERCC1 in non–small-cell lung cancer and cisplatin-based adjuvant chemotherapy. N Engl J Med, 2006, 355: 983–991.
4. Micalizzi DS, Farabaugh SM, Ford HL. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. J Mammary Gland Biol Neoplasia, 2010, 15: 117–134.
5. Thiery JP, Acloque H, Huang RY, *et al*. Epithelial-mesenchymal transitions in development and disease. Cell, 2009, 139: 871–890.
6. Rho JK, Choi YJ, Lee JK, *et al*. Epithelial to mesenchymal transition derived from repeated exposure to gefitinib determines the sensitivity to EGFR inhibitors in A549, a non-small cell lung cancer cell line. Lung Cancer, 2009, 63: 219–226.
7. Ceppi P, Mudduluru G, Kumarswamy R, *et al*. Loss of miR-200c expression induces an aggressive, invasive, and chemoresistant

phenotype in non-small cell lung cancer. Mol Cancer Res, 2010, 8: 1207–1216.
8. Che Y, Xu J, Su S, *et al.* Reversal effect of recombinant human Endostatin on cisplatin resistance in A549/DDP human lung adenocarcinoma cells *in vitro*. Chinese-German J Clin Oncol, 2013, 12: 509–515.
9. Sarkar FH, Li Y, Wang Z, *et al*. Pancreatic cancer stem cells and EMT in drug resistance and metastasis. Minerva Chir, 2009, 64: 489–500.
10. Foroni C, Broggini M, Generali D, *et al.* Epithelial-mesenchymal transition and breast cancer: role, molecular mechanisms and clinical impact. Cancer Treat Rev, 2012, 38: 689–697.
11. Song WA, Zhou NK, Wang W, *et al.* Survival benefit of neoadjuvant chemotherapy in non-small cell lung cancer: an updated meta-analysis of 13 randomized control trials. J Thorac Oncol, 2010, 5: 510–516.
12. Maseki S, Ijichi K, Tanaka H, *et al.* Acquisition of EMT phenotype in the gefitinib-resistant cells of a head and neck squamous cell carcinoma cell line through Akt/GSK-3β/snail signalling pathway. Br J Cancer, 2012, 106: 1196–1204.
13. Bonavida B, Baritaki S. The novel role of Yin Yang 1 in the regulation of epithelial to mesenchymal transition in cancer via the dysregulated NF-κB/Snail/YY1/RKIP/PTEN Circuitry. Crit Revi Oncog, 2011, 16: 211–226.
14. Zhuo W, Wang Y, Zhuo X, *et al*. Knockdown of Snail, a novel zinc finger transcription factor, via RNA interference increases A549 cell sensitivity to cisplatin via JNK/mitochondrial pathway. Lung Cancer, 2008, 62: 8–14.
15. Güngör C, Zander H, Effenberger KE, *et al*. Notch signaling activated by replication stress-induced expression of midkine drives epithelial-mesenchymal transition and chemoresistance in pancreatic cancer. Cancer Res, 2011, 71: 5009–5019.
16. Zhuo WL, Wang Y, Zhuo XL, *et al*. Short interfering RNA directed against TWIST, a novel zinc finger transcription factor, increases A549 cell sensitivity to cisplatin via MAPK/mitochondrial pathway. Biochemi Biophys Res Communi, 2008, 369: 1098–1102.
17. Han Z, Hong L, Han Y, *et al*. Phospho Akt mediates multidrug resistance of gastric cancer cells through regulation of P-gp, Bcl-2 and Bax. J Exp Clin Cancer Res, 2007, 26: 261–268.
18. Li W, Liu C, Tang Y, *et al*. Overexpression of Snail accelerates adriamycin induction of multidrug resistance in breast cancer cells. Asian Pac J Cancer Prev, 2011, 12: 2575–2580.
19. Zhu K, Chen L, Han X, *et al.* Short hairpin RNA targeting Twist1 suppresses cell proliferation and improves chemosensitivity to cisplatin in HeLa human cervical cancer cells. Oncol Rep, 2012, 27: 1027–1034.
20. Saxena M, Stephens MA, Pathak H, *et al*. Transcription factors that mediate epithelial-mesenchymal transition lead to multidrug resistance by upregulating ABC transporters. Cell Death Dis, 2011, 2: e179.

《中德临床肿瘤学杂志》2014年征稿启事

《中德临床肿瘤学杂志》是由中华人民共和国教育部主管，华中科技大学同济医学院主办的医学肿瘤学学术期刊（全英文月刊），已先后被中信所科技核心数据库、中国期刊全文数据库、万方数据资源系统数字化期刊群、维普资讯网科技期刊数据库、中国学术期刊综合评价数据库、EMBASE、Index Copernicus等国内外重要检索系统收录。国际、国内刊号为：ISSN 1610-1979（纸质版），1613-9089 (网络版)；CN 42-1654/R，邮发代号：38-121。

本刊主要刊登世界各国作者，特别是中国作者在肿瘤学领域的优秀科研成果和临床诊疗经验，包括与临床肿瘤学密切相关的基础理论研究等成果，并全文以英语发表，在国内外公开发行。辟有述评、专家笔谈、论著、临床研究、实验研究、综述、病例报道、人物专栏等栏目。

本刊具有编审效率高、出版周期短、学术价值高、临床实用性强、印刷精美等特点。承诺将一如既往地以广大作者为依托，积极为作者和读者服务，严格做到对所有来稿处理及时、审稿认真、退修详细、发稿迅速。对具有国际领先水平的创新科研成果及国家重点项目开辟“绿色通道”，审稿迅速，刊登及时。

欢迎全国各级肿瘤学医务工作者踊跃投稿、组稿！

《中德临床肿瘤学杂志》2014年重点专栏报道计划如下：

1，肺癌；2，肝癌；3，胰腺肿瘤；4，胃肠肿瘤；5，乳腺肿瘤；6，甲状腺癌；7，骨肿瘤；8，泌尿生殖系肿瘤；9，脑肿瘤；10，血液系统疾病；11，妇科肿瘤；12，耳鼻喉科肿瘤；13，皮肤肿瘤；14，肿瘤诊断学（特别是肿瘤影像诊断学）；15，肿瘤化疗；16，肿瘤放疗；17，肿瘤转化医学；18，肿瘤心理学；19，其他。

敬请您关注！具体投稿和联系方式请参见杂志版权页。

《中德临床肿瘤学杂志》编辑部