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c-Met and RON expression levels in endometrial adenocarcinoma tissue and their relationship with prognosis

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Summary

Objective: To investigate the potential relevance of c-Met and RON gene expression in patients with adenocarcinoma of the endometrium and analyze the relationships among the c-Met and RON expression, clinicopathological characteristics, and patient survival. *Materials and Methods:* The study included 60 cases diagnosed with endometrial adenocarcinoma with more than five-years follow-up. Total RNA from formalin-fixed paraffin-embedded tissues of 60 adenocarcinomas of the endometrium and normal endometrium tissues were isolated for c-Met and RON quantitative analysis by real-time real-time polymerase chain reaction (RT-PCR). *Results:* The c-Met and RON expression in endometrial adenocarcinoma was significantly higher than that in normal endometrial tissues ($p < 0.01$), with average up-regulated levels of 3.94 ± 1.88 and 2.74 ± 0.88, respectively. Moreover, high c-Met expression was significantly correlated with the histological stage ($p = 0.017$), and high RON expression was related to histological stage ($p = 0.035$), muscle invasion ($p = 0.006$), and lymph node metastasis ($p = 0.018$). Multivariate Cox regression analysis revealed that the co-expression of c-Met and RON was an independent prognostic factor for adenocarcinoma of the endometrium and was significantly associated with decreased overall survival (HR = 3.571, $p = 0.014$). *Conclusion:* The co-expression of c-Met and RON is associated with a poor prognosis in endometrial adenocarcinoma and is an independent prognostic marker for endometrioid adenocarcinoma.

Key words: c-Met; RON; Endometrioid adenocarcinoma; Prognosis.

Introduction

Studies of epithelial tumors have demonstrated that receptor tyrosine kinases (RTKs) of transmembrane proteins play a basic role in cell growth, differentiation, and survival, and are important in tumor occurrence and development. c-Met and RON (recepteur d'origine nantais) belong to the MET proto-oncogene family, a distinct subfamily of RTKs. c-Met protein can be activated by its ligand hepatocyte growth factor receptor. c-Met can be conformationally changed by their receptor intracellular tyrosine kinase domain, and participate in multiple cellular processes, including proliferation, differentiation, morphogenesis, and infiltration [1]. RON can induce cell differentiation and development of epithelial tumors [2, 3]. Recent studies have shown that c-Met and RON are both separately expressed and co-expressed in various tumors [4, 5] and interact in several epithelial tumors, leading to tumorigenesis. Therefore, understanding tyrosine kinase expression will help to define the role of c-Met and RON in carcinogenesis and their potential as molecular targets for tumor therapy.

Endometrial carcinoma is a common gynecological malignant tumor, accounting for 20–30% of all malignant tumors of the female genital tract. For all endometrial carcinomas, the incidence of endometrial adenocarcinoma is 80–90%. The present study evaluated the c-Met and RON expression levels in endometrial adenocarcinoma and normal endometrial tissue by real-time polymerase chain reaction (RT-PCR), and explored the relationship between c-Met and RON expression and clinicopathological features in endometrial adenocarcinoma.

Materials and Methods

Test specimens

Formalin-fixed paraffin-embedded (FFPE) archived samples of endometrial adenocarcinoma from 60 cases were collected from January 2005 to December 2006 in Wenzhou City, at the Traditional Chinese and Western Medicine Hospital Pathology Department, and FFPE archived samples from 20 cases with normal endometrium were used for paraffin tissue RNA extraction. Simultaneously, the authors collected from March 2010 to October 2011 in Wenzhou Hospital of Integrated Chinese and Western medicine for "endometrial adenocarcinoma" line operation of fresh specimens of 15 cases of endometrial cancer, because of "uterine leiomyoma" line of hysterectomy in ten cases of normal endometrium. Following resection, fresh tissue was immediately frozen in liquid nitrogen, and then stored at -80°C. Fresh specimens tissue for RNA extraction control with paraffin. All cases with FFPE tissue had complete clinical pathological data available and were followed up for more than five

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years. The 60 FFPE cases of endometrial carcinoma underwent an operation for staging and pathological grading by two experienced pathologists using the 2000 International Federation of Gynecology and Obstetrics (FIGO) pathological staging criteria. Fifteen cases were classified as pathological Stage G1, 30 as Stage G2, and 15 as Stage G3. Twenty-eight cases were clinically classified as Stages 0–I, 14 cases as Stage II, and 18 cases as Stage III. Thirty-six cases had no myometrial invasion or depth of myometrial invasion ≤ 1/2, 24 cases had a depth of myometrial invasion >1/2, 47 cases had no lymph node metastasis, and 13 cases showed metastasis. Patients received no radiotherapy, chemotherapy, or hormone therapy. Clinical and pathological data, as well as follow-up data, for the 60 cases with endometrial adenocarcinoma are presented in Table 1.

Follow-up

All cases were followed up by telephone. Those who could not be contacted at the fourth attempt were followed-up through local medical institutions or personnel inquiries. The deadline was July 1st, 2011. During the five-year follow-up period, six cases (10%) were lost and 14 died.

Real-time quantitative reverse transcription–PCR

FFPE RNA extraction was performed as follows: (1) FFPE samples were dewaxed by adding xylene on slices, incubating the samples for ten minutes at room temperature, followed by overturning and blending to melt the paraffin. Residual xylene was removed by washing twice with absolute ethyl alcohol, and then the lamellar precipitate was air-dried. (2) RNA was then extracted and purified using the RecoverAllTM total nucleic acid isolation kit for FFPE. (3) RNA was dissolved by adding 60 μL of elution solution to purified RNA. Of the purified RNA solution, 20 μl was reserved for detection of RNA quality and reverse transcription, and the remainder was stored at -80°C.

RNA was extracted by first liquid-nitrogen grinding fresh tissue into a powder, addition of TRIzol, and then extracting the RNA according to the TRIzol instruction manual.

Primers were designed using the Primer 3 software, accessed online. Primers were designed with a GC content of 40–60% and a product size of 89–203 bp. Primers were then evaluated using Oligo 6.0 software. Primers contained intron sequences to reduce interference from genomic DNA and were synthesized.

The RT–PCR primer pairs used for c-Met and RON expression were as follows:

c-Met (212 bp): upstream, 5′-CAGGCAGTGCAGCATGTAGT-3′/downstream, 5′-GATGATTCCCTCGGTCAGAA-3′; RON (292 bp): upstream, 5′-TGGGGACCACCTACTCTTTG-3′/downstream, 5′- GAGCCAGGACACTCCTTCTG-3′; GAPDH (314 bp): upstream, 5′-GGTCGGAGTCAACGGATTTG-3′/downstream, 5′-ATGAGCCCCAGCCTTCTCCAT-3′. The 25-μL PCR reaction contained 12.5 μL of 2× QuantiTect SYBR Green PCR buffer, 10 μM of primers and 1 μL or 2 μL of cDNA sample (300 ng), RNA enzyme inactivation 8.5 μL. Thermal cycle conditions were as follows: 94°C for five minutes; 40 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 60 seconds, and 72°C for ten minutes.

After selecting the appropriate baseline, the crossover point of the fluorescence curves and baseline was termed the Ct (cycle threshold) value. The smaller the Ct value under the same baseline, the earlier the fluorescence signal detected, reflecting a higher number of gene copies. The Ct values of the target and reference genes were calculated by the 2− ΔΔ Ct method [6], and GAPDH was applied as control. Compared with normal endometrial tissue, relative c-Met mRNA expression levels were calculated using the following formula: folds = 2−ΔΔ Ct, while Δ Ct = (Ct Δ which gene − Ct endogenous reference gene) study group −

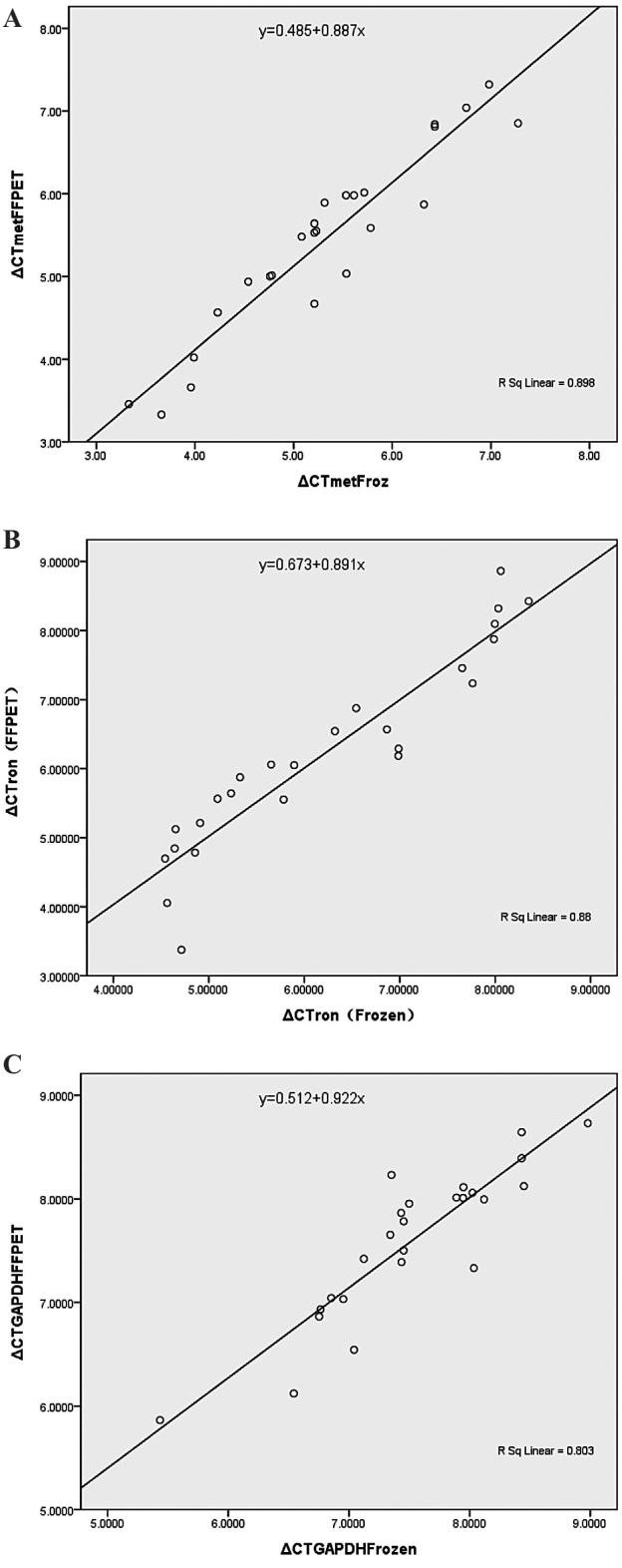

Figure 1. — Comparison of c-Met and RON expression using total RNA extracted from matched samples of formalin-fixed paraffin-embedded tissue (FFPET) and frozen tissue. Comparison of theΔCt values for paired FFPET and frozen tissue for (A) c-Met, (B) RON, and (C) GAPDH.

Table 1. — *Distributions of c-Met and RON gene expression and biological indicators.*

Clinical characteristics		Number of cases	c-Met mRNA $x \pm s$	t	p value*	RON mRNA $x \pm s$	t	p value*
Age (years)	<50	38	3.457 ± 0.736	1.542	0.417	2.887 ± 0.466	1.021	0.312
	≥50	22	3.787 ± 0.624			2.752 ± 0.451		
Menopause	Yes	15	3.639 ± 0.924	1.003	0.321	2.832 ± 0.638	0.448	0.656
	No	45	3.411 ± 0.689			2.671 ± 0.654		
Clinical stage	I, II	42	3.698 ±0.892	1.361	0.180	2.926 ± 0.611	1.949	0.047
	III*	18	3.397 ± 0.664			2.590 ± 0.605		
Muscle invasion	<1/2	36	3.546 ± 0.841	0.697	0.489	2.571 ± 0.591	2.849	0.006
	≥1/2	24	3.397 ± 0.665			3.011 ± 0.654		
Histological grade	I	15	3.323 ± 0.629	2.484	0.017	2.643 ± 0.563	2.164	0.035
	II, III	45	3.852 ± 0.841			3.008 ± 0.629		
Lymph node status	Metastasis	13	3.615 ± 0.788	1.059	0.295	2.981 ± 0.637	2.439	0.018
	No metastasis	47	3.397 ± 0.665			2.593 ± 0.483		

*No Stage IV patients were identified.

(Ct target gene− Ct endogenous reference gene) control group indicates the expression of c-Met mRN in endometrioid adenocarcinoma relative to that in normal endometrial tissue. The relative quantity of RON mRNA was calculated in the same way.

Statistical analysis

Data were analyzed using the SPSS software, version 15. The results are expressed as X ± s, and group comparisons were performed using independent sample tests. A value of $p < 0.05$ was deemed to indicate statistical significance. Relationships between c-Met and RON expression and clinicopathological features of endometrioid adenocarcinoma were assessed by the $\chi 2$ test. The Kaplan–Meier method and Cox regression analysis were used to evaluate relationships among c-Met and RON expression, clinicopathological features of endometrioid adenocarcinoma, and prognosis.

Results

Reliability of RT-PCR for detection of c-Met and RON expression in paraffin-embedded tissues

The ratios of the absorbances at 260 and 280 nm (A260/A280) of overall RNA extracted from paraffin-embedded endometrial carcinoma tissue were 1.75–1.95; there was no significant difference between the two groups ($p > 0.05$). Levels of c-Met and RON expression were correlated (R2 = 0.898 and 0.88, respectively; $p < 0.01$, linear regression; Figures 1A and 1B). In paraffin-embedded endometrial adenocarcinoma tissue, Δ Ctc-Met = 5.34 ± 1.13 whereas Δ Ctc-Met = 5.23 ± 1.01 in the corresponding fresh tissue.In paraffin-embedded endometrial adenocarcinoma tissue, Δ CtRON = 5.70 ± 1.85 whereas Δ CtRON = 6.21 ± 1.53 in the corresponding fresh tissue.

GAPDH RNA expression levels in the two types of tissues were also related (Figure 1C).

Relationship between relative c-Met and RON expression levels in endometrial carcinoma and clinicopathological features

Relative mRNA expression levels of c-Met and RON in endometrial adenocarcinoma tissue were calculated to be 3.94 ± 1.88 and 2.74 ± 0.88, respectively. These levels followed a normal distribution ($p > 0.05$) and were significantly higher than those in normal endometrial tissue (0.672 ± 0.098 for endometrial adenocarcinoma tissue; 0.512 ± 0.109 for normal endometrial tissue; $p < 0.01$). c-Met mRNA expression levels in endometrial adenocarcinoma was related to pathological staging ($p = 0.017$). RON expression levels in patients were related to pathological staging ($p = 0.035$), depth of myometrial invasion (p = 0.006), and lymph node metastasis ($p = 0.018$; Table 1).

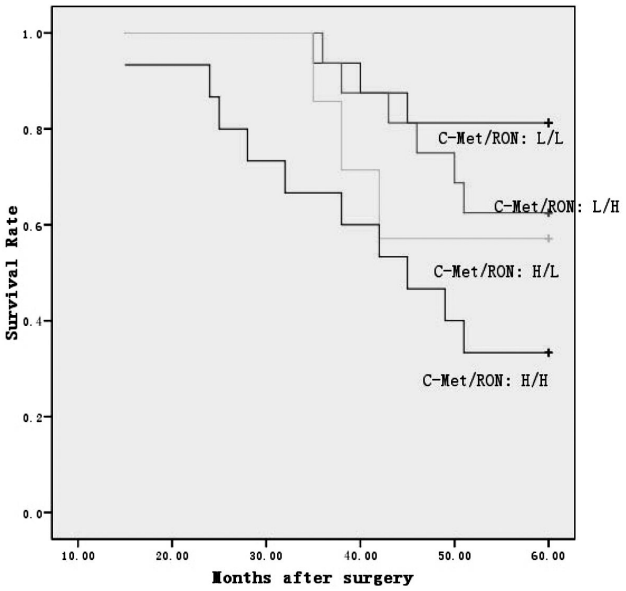

Figure 2. — The prognostic significance of c-Met and RON expression in endometrial carcinoma patients. Patients who co-expressed c-Met and RON had a significantly worse survival compared with patients who had single-receptor-positive tumors or no receptor expression.

Table 2. — *Prognostic significance of biological indicators and c-Met and RON expression for endometrial carcinoma patients (univariate Cox regression analysis).*

Variable	Subset	Hazard ratio (95% CI)	p*
Age	<50 *vs*. ≥50	1.139 (0.431–4.040)	0.08
Histology grade	I *vs*. II, III	3.562 (1.473–7.892)	0.012
Clinical stage	I, II *vs*. III	1.151 (0.336–3.617)	0.809
Lymph nodes	− *vs*. +	2.314 (1.123–6.328)	0.331
Muscle invasion	<1/2 *vs*. ≥1/2	1.380 (0.500–3.807)	0.533
c-Met	Low *vs*. high	1.335 (0.763–6.323)	0.046
RON	Low *vs*. high	3.279 (1.892–8.921)	0.014
c-Met and RON	Low, Low *vs*. high, high	2.982 (1.763–9.873)	0.004

Abbreviations: − *vs*. +: negative *vs*. positive; CI: confidence interval; multivariate Cox regression, stepwise forward LR.

Table 3. — *Prognostic significance of biological indicators and c-Met and RON expression for endometrial carcinoma patients (multivariate Cox regression analysis).*

Variable	Subset	Hazard ratio (95% CI)	p*
Histology grade	I *vs*. II, III	2.471 (2.219–8.543)	0.016
c-Met, RON	Low, Low *vs*. High, High	3.571 (1.291–9.875)	0.014
Age	<50 *vs*. ≥50		0.628
Clinical stage	I, II *vs*. III		0.315
Lymph nodes	− *vs*. +		0.106
Muscle invasion	<1/2 *vs*. ≥1/2		0.065
c-Met	Low *vs*. High		0.571
RON	Low *vs*. High		0.461

Abbreviations: − *vs*. +, negative *vs*. positive; CI: confidence interval; multivariate Cox regression, stepwise forward LR.

Relative expression of c-Met and RON in endometrial carcinoma and prognosis

The five-year survival rate of the 60 cases was 74.07% (40/54; Figure 2). Kaplan–Meier survival analysis showed that the five-year survival rate in the high c-Met and RON co-expression group was significantly higher than that of high or low expression separately ($p < 0.01$; Figure 2).

Cox single regression analysis showed that pathological grading, abnormal expression of c-Met and RON, and c-Met /RON co-expression in endometrial carcinoma were poor prognostic indicators (Table 2). Cox proportional hazards model analysis showed that pathological grading and high expression of c-Met /RON in endometrial carcinoma were independent prognostic factors [$p = 0.014$, hazard ratio (HR) = 3.571; 95% confidence interval (CI): 1.291–9.875]. Additionally, pathological grading (Table 3) was an independent factor prognostic of endometrial cancer.

Discussion

Since 1988, when Rupp and Locker first extracted RNA from paraffin blocks [7], RNA studies have not been dependent on fresh or frozen probes. Subsequently, several studies have found that miRNAs in total RNA extracted from paired frozen cells and formaldehyde-fixed, paraffin-embedded cells were comparable [8]. Additionally, the application of RNA extraction from paraffin-embedded tissue in molecular biology has been extended further. The present study extracted RNA from FFPE tissue and fresh tissue of endometrial adenocarcinoma, respectively, and detected the expression of c-Met and RON by RT-PCR. The results showed there were significant correlations between the two types of tissue. (R2 = 0.898 and 0.88, $p < 0.01$), which signifies that the detection of c-Met and RON expression by RT-PCR is sufficiently reliable. The results also showed that routine paraffin-embedded archived tissue may be used for mRNA retrospective studies, so that the resources of the Department of Pathology are utilized fully.

Research has shown that c-Met positive cancer cells have higher ability of invasion and remote metastasis, and RON has the same impact as c-Met does. Additionally, the role of the interaction between c-Met and RON in tumorigenesis and progression is supported by the results of an in vitro tumor-related study [9]. The relationship between c-Met and endometrial adenocarcinoma is usually investigated by immunohistochemistry in a semiquantitative manner; however, few such studies have been performed. To-date, no study has reported the relationship between the RON gene and endometrial adenocarcinoma. Additionally, the role of both genes in development of endometrial adenocarcinoma is unclear.

The present study detected the expressions of c-Met and RON in FFPE of 60 cases of endometrial adenocarcinoma and 20 cases of normal endometrium. The present results showed c-Met and RON expression levels in endometrial adenocarcinoma tissues was significantly higher than those in normal endometrial tissue ($p < 0.01$). Significant correlations were found between abnormal RON expression and histological stage, depth of myometrial invasion, and lymph node metastasis in the first consultation. Abnormal c-Met expression was related to pathological staging. There were significant correlations between c-Met and RON expression and pathological stages and histological grades. The later the pathological staging, the lower the differentiation and the higher the expression of c-Met and RON. The

two expression levels were significantly correlated with the depth of myometrial invasion and the status of metastasis when initial diagnosis is established ($p < 0.05$), indirectly confirming that these genes may promote development of endometrial adenocarcinoma. High expression of the tyrosine kinases c-Met and RON may be a relatively late event in endometrial adenocarcinoma and may be related to invasion and metastasis [4]. Therefore, assessment of c-Met and RON expression in endometrial adenocarcinoma provides a basis for further treatment. The present results are consistent with the findings that different subtypes of the same ErbB receptor family interact and are related to poor prognosis [3]. Cheng *et al*. [5] reported co-expression of c-Met and RON to be an important indicator of poor prognosis in bladder cancer and related to tumor recurrence. Through Cox multiple regression analysis, the present study indicated that high expression of c-Met and RON ($p = 0.014$) and pathological grade ($p = 0.016$) were indicators of poor prognosis in endometrial adenocarcinoma. High level of co-expression of c-Met and RON may thus represent a "molecular prognostic index" in endometrial adenocarcinoma.

In summary, up-regulated c-Met and RON expression levels were closely related to the occurrence, development, and prognosis of endometrial adenocarcinoma. Simultaneous detection of the expression of both genes provides diagnostic and prognostic information regarding endometrial adenocarcinoma. However, further research is needed to elucidate the mechanism underlying the role of c-Met and RON in the development of endometrial adenocarcinoma. In terms of their role in carcinogenesis, c-Met and RON may represent novel target molecules for gene therapy as a future treatment option for endometrial adenocarcinoma.

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