

## ***RUNX3* copy number predicts the development of UC-associated colorectal cancer**

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**Abstract.** *RUNX3* is a tumour suppressor gene that plays an important role in the development of various cancers. The present study aimed to compare *RUNX3* mRNA expression levels and DNA copy numbers in the non-neoplastic rectal mucosa between ulcerative colitis (UC) patients with and without UC-associated colorectal cancer (UC-Ca). We further aimed to build a predictive model of the development of UC-Ca based on the *RUNX3* DNA copy number. *RUNX3* mRNA expression levels were quantified by RT-PCR. The hypermethylation and DNA copy number of *RUNX3* were also determined. Thirty-five UC patients were examined, 17 of whom had UC-Ca (UC-Ca group) and 18 who did not (UC-NonCa group). The UC-Ca group had significantly lower m*RUNX3* expression levels and smaller DNA copy numbers than the UC-NonCa group ( $p=0.04$ ,  $p=0.0016$ , respectively). *RUNX3* expression levels correlated with DNA copy numbers. Classification of the UC-Ca and UC-NonCa group based on DNA copy number gave an accuracy of 82.9%. *RUNX3* expression levels in the non-neoplastic rectal mucosa was significantly decreased in the UC-Ca group and it is

suggested that this was attributable to the decrease in *RUNX3* DNA copy number. The present predictive model may be useful in the selection of high risk UC-Ca patients and to improve the efficacy of surveillance colonoscopy. The present study suggests that *RUNX3* might play an important role in the development of UC-Ca.

### **Introduction**

Patients with long-standing ulcerative colitis (UC) have an increased risk of developing colorectal cancer. This risk increases with the duration of the disease, and the estimated cumulative risk of UC-associated colorectal cancer (UC-Ca) 30 years after the onset of UC has been reported by meta-analysis to be 18% (1). Therefore, for the early detection of neoplastic lesions, surveillance colonoscopy is recommended for patients whose duration of the disease is 7-8 years or more (2-4). However, to further improve the efficacy of surveillance, new markers to predict the development of UC-Ca are urgently needed.

Previous studies have shown that patients with UC-Ca have widespread genetic alterations in the non-neoplastic colonic mucosa, suggesting that such changes might be effective predictors of UC-Ca development (5-8). Indeed, we previously demonstrated this by DNA microarray gene expression analysis (9). However, to date, specific molecular markers that can be used in clinical settings have not been established.

*RUNX3* belongs to the Runt domain family of transcription factors, and is involved in T-cell differentiation, the TGF- $\beta$ -induced tumour suppressor pathway (10,11) and is a known tumour suppressor gene in gastric cancer (12). Previous studies

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reported that alterations in *RUNX3* gene expression were present in various types of cancer including breast, lung, hepatocellular, prostate, bile duct, pancreatic and colon cancers (10-12). Studies have also shown that *RUNX3* gene expression levels are influenced by the hypermethylation of CpG islands in the *RUNX3* promoter region (13-19). On the other hand, a recent report suggested a role for *RUNX3* in UC susceptibility, the development of colitis and gastric mucosal hyperplasia (20,21). Weersma *et al* showed that *RUNX3* mRNA expression is increased in the colonic mucosa of UC patients compared with controls (20).

In our previous microarray analysis, *RUNX3* was expressed at lower levels in the non-neoplastic mucosa of patients with UC-Ca compared to those without (9). However, microarray analysis is known to lack reproducibility as a means of quantitative analysis of gene expression. In order to accurately validate gene expression levels, it requires verification by an alternative quantitative approach, such as real-time PCR (22,23). Another problem with microarray analysis is the large number of genes used in prediction models. In our previous model, gene expression levels of 72 genes were examined to predict the development of UC-Ca. Because of these problems, the use of microarrays in clinical practice remains limited, and recent studies have shown that RT-PCR analyses of a small number of selected genes are useful in predicting the outcome of patients in various diseases (22,23).

Therefore, in the present study, we aimed to confirm the decreased expression of *RUNX3* by RT-PCR in patients with UC-Ca. We also analysed possible mechanisms for decreased *RUNX3* expression by examining the hypermethylation of CpG islands in the promoter region, and *RUNX3* DNA copy numbers. Lastly, we aimed to use this copy number to establish a predictive model of patients with UC-Ca.

## Materials and methods

**Patients and samples.** Thirty-five UC patients were examined. Informed consent was obtained from all patients for the collection of specimens, and the study protocol was approved by the local Ethics Committee. All UC patients had total colitis and their disease had persisted for more than seven years; they were therefore considered to be at high risk of cancer and/or dysplasia development. Among the 35 UC patients, 17 had UC-associated adenocarcinoma (UC-Ca group) and 18 had no neoplastic lesions (UC-NonCa group). Patient characteristics are shown in Table I.

In all UC-Cases, specimens were obtained from non-neoplastic rectal mucosa for DNA and RNA extraction (Fig. 1). Samples were taken either from surgically resected specimens or during surveillance colonoscopic examination. Samples were snap-frozen in liquid nitrogen and stored at -80°C until use. Paralleled tumour specimens were formalin-fixed and paraffin-embedded for histological examination as described previously (24). Microscopic examination of rectal mucosa in UC patients verified that no neoplastic cells were present in any of the samples.

**RNA isolation and RT-PCR.** Total RNA was isolated from frozen samples using the RNeasy Mini kit (Qiagen, Valencia, CA). Gene expression levels were determined using TaqMan

real-time PCR (Applied Biosystems, Foster City, CA) as described previously (Fig. 1) (25). First-strand cDNA was synthesized from total RNA using the High Capacity cDNA Archive kit (Applied Biosystems) in 50  $\mu$ l reactions using cDNA samples for TaqMan real-time PCR analysis according to the manufacturer's instructions. cDNA (10 ng/ $\mu$ l) was added to 9.15  $\mu$ l RNase-free water, 12.5  $\mu$ l 2 x TaqMan Universal PCR Master Mix (Applied Biosystems) and 1.25  $\mu$ l 20 x Primer Probe mix. ACTB (actin,  $\beta$ ) was used as the endogenous control gene (Applied Biosystems). Assay IDs were Hs00231709\_m1 and Hs99999903\_m1 for *RUNX3* and ACTB, respectively. PCR amplification was carried out using the Prism 7900HT Sequence Detection System (Applied Biosystems) under the following thermal cycler conditions: 2 min at 50°C and 10 min at 94.5°C for 40 cycles (30 sec at 97°C and 1 min at 59.7°C). Relative *RUNX3* gene expression was calculated by comparing the  $\delta$  CT values as previously described (26).

**Genomic DNA preparation and *RUNX3* methylation-specific PCR.** Hypermethylation of the promoter region of the *RUNX3* gene was determined by methylation-specific PCR. Genomic DNA was extracted using the QIAAMP DNA mini kit (Qiagen), according to the manufacturer's instructions. Bisulfite conversion of DNA samples was carried out essentially as described and was based on the principle that treatment of DNA with bisulfite would result in the conversion of unmethylated cytosine residues into uracil. Methylated cytosine residues, on the other hand, would remain unchanged (19). Thus, the DNA sequences of methylated and unmethylated genomic regions following bisulfite conversion would be different and distinguishable by sequence-specific PCR primers.

Bisulfite conversion was carried out using an EZ DNA methylation kit (ZYMO research, Orange, CA). DNA (2  $\mu$ g) was treated with sodium bisulfite following the manufacturer's recommendations and resuspended in a total volume of 10  $\mu$ l. Hypermethylated DNA treated with Sss I and normal genomic DNA were used as methylation-positive and -negative control samples, respectively, in the following PCR assay: sense and antisense primers for the bisulfite-converted methylated sequence were: m*RUNX3*-F (5'-TTA CGA GGG GGG GTC GTA CGC GGG-3') and m*RUNX3*-R (5'-AAA ACG ACC GAC GCG AAC GCC TCC-3') respectively. Sense and antisense primers for the bisulfite-converted unmethylated sequence were: u*RUNX3*-F (5'-TTA TGA GGG GTG GTT GTA TGT GGG-3') and u*RUNX3*-R (5'-AAA ACA ACC AAC ACA AAC ACC TCC-3'), respectively (19).

Real-time PCR was performed on a PRISM 7900 sequence detector (Applied Biosystems) using a QuantiTect SYBR Green kit (Qiagen). Conditions for quantitative PCR were as follows: one cycle at 94°C for 15 min, 45 cycles at 94°C for 20 sec, 56°C for 20 sec, and 70°C for 30 sec. The methylation status was judged from the calculated delta Ct value derived from the methylated and unmethylated sequence primer CT values.

**Genomic DNA preparation and quantitative real-time PCR for determination of the *RUNX3* DNA copy number.** The *RUNX3* DNA copy number was determined by real-time PCR.

Table I. Characteristics of patients.

| Characteristic               | CUC-Ca group<br>(n=17) | UC-NonCa group<br>(n=18) | P-value |
|------------------------------|------------------------|--------------------------|---------|
| Age at biopsy (years)        |                        |                          | NS      |
| Mean, SD                     | 54.3 (34-70), 10.5     | 48.8 (25-70), 11.8       |         |
| Age at UC diagnosis (years)  |                        |                          | NS      |
| Mean, SD                     | 36.6 (15-52), 13.1     | 31.4 (15-55), 9.8        |         |
| Gender                       |                        |                          | NS      |
| Male                         | 12                     | 10                       |         |
| Female                       | 5                      | 8                        |         |
| Duration of disease (years)  |                        |                          | NS      |
| Mean, SD                     | 17.7 (9-29), 6.8       | 14.8 (9-25), 6.9         |         |
| Extent of UC                 |                        |                          | NS      |
| Total colitis                | 15                     | 16                       |         |
| Left-sided colitis           | 2                      | 2                        |         |
| Proctosigmoiditis            | 0                      | 0                        |         |
| Proctitis                    | 0                      | 0                        |         |
| Inflammation                 |                        |                          | NS      |
| None                         | 4                      | 3                        |         |
| Mild                         | 12                     | 12                       |         |
| Moderate                     | 1                      | 3                        |         |
| Severe                       | 0                      | 0                        |         |
| Medication                   |                        |                          | NS      |
| Mesalamine                   | 12                     | 11                       |         |
| Corticosteroids <sup>3</sup> | 5                      |                          |         |
| 6MP/AZA                      | 2                      | 2                        |         |
| CSA                          | 0                      | 0                        |         |
| PSC                          |                        |                          | NS      |
| Present                      | 0                      | 0                        |         |
| Not present                  | 17                     | 18                       |         |

SD, standard deviation; UC, ulcerative colitis; 6MP/AZA, 6-mercaptopurine/azathioprine; CSA, cyclosporin A; PSC, primary sclerosing cholangitis.

Genomic DNA was extracted from samples using the QIAAMP DNA mini kit (Qiagen) as before. Quantitative real-time PCR was performed on a PRISM 7900 sequence detector with a QuantiTect SYBR Green kit as before. We quantified the DNA by comparing the target locus to the reference Line-1, a repetitive element for which copy numbers per haploid genome are similar among all human normal and neoplastic cells. The relative target copy number level was also normalized to normal human genomic DNA as a calibrator. Copy number changes of the target gene relative to Line-1 and the calibrator were determined using the formula  $(T_{\text{target}}/T_{\text{Line-1}})/(C_{\text{target}}/C_{\text{Line-1}})$ , where  $T_{\text{target}}$  and  $T_{\text{Line-1}}$  are quantities from tumour DNA using target and Line-1, and  $C_{\text{target}}$  and  $C_{\text{Line-1}}$  are quantities from the calibrator using target and Line-1. PCRs for each primer set were performed in triplicate or more, and mean values and standard deviations reported (19,26).

Conditions for quantitative PCR were as above. At the end of the PCR reaction, samples were subjected to melting analysis to confirm amplicon specificity. PrimerExpress software, ver.2.0 was used to design the primers to span a 200 bp non-repetitive region, and primers were then synthesized by Operon Biotechnologies Inc. (Tokyo, Japan). The primer set was subsequently compared with the human genome using a basic local alignment search tool algorithm to determine its uniqueness. Primer sequences for *RUNX3* are: Forward primer (intron 3), 5'-CCAACCACCTGCCTCT ATTCC-3'; Reverse primer (exon 4), 5'-TTGGTGAACACA GTGATGGTCA-3'.

*Prediction of UC-associated neoplasm development by RUNX3 mRNA expression and DNA copy number.* Areas under the receiver operator characteristic (ROC) curve (AUCs) were

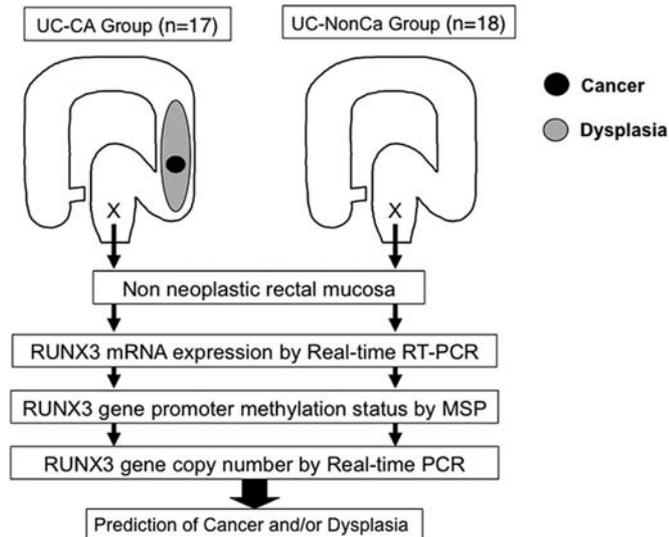


Figure 1. Sample collection and data analysis procedure. Samples were obtained from non-neoplastic rectal mucosa for RNA and DNA extraction. *RUNX3* mRNA expression level, hypermethylation of *RUNX3* promoter region and *RUNX3* DNA copy number were compared between UC-Ca and UC-NonCa groups.

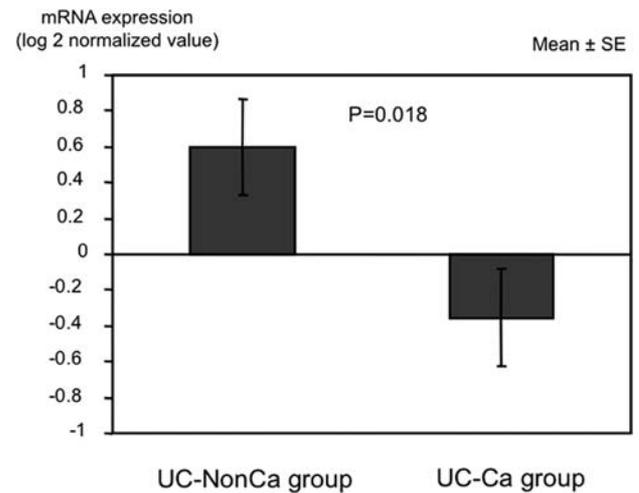


Figure 2. Mean mRNA expression of *RUNX3*. UC-NonCa group showed significantly higher *RUNX3* mRNA expression level than UC-Ca group ( $p=0.018$ ). Vertical axis shows normalized *RUNX3* mRNA expression level. (A bar indicates standard deviation).

Table II. Methylation status of *RUNX3* gene promoter region.

|                       | No. of cases with hypermethylation | No. of cases without hypermethylation | Frequency of hypermethylation (%) |
|-----------------------|------------------------------------|---------------------------------------|-----------------------------------|
| UC-Ca group (n=17)    | 0                                  | 17                                    | 0                                 |
| UC-NonCa group (n=18) | 0                                  | 18                                    | 0                                 |

calculated according to expression level or copy number for the ability of the logistic regression models to differentiate between UC-Ca and UC-NonCa patients. The predictive accuracy was determined by measuring the specificity, sensitivity, and area under the ROC curve. A predictive model with an ROC of  $\geq 0.7$  is considered to have good discrimination, and an area under the ROC curve of 0.5 is equivalent to a 'coin toss'.

**Statistical analysis.** Associations between *RUNX3* gene expression, promoter methylation, DNA copy number and clinical outcomes were tested using unpaired t-tests, generalized logistic model analysis or Fisher's exact test, as appropriate. Data were analyzed using JMP ver 5.0 software (SAS Institute, Tokyo).

## Results

**mRNA expression level by RT-PCR analysis.** Gene expression levels of *RUNX3* were significantly lower (1.9-fold) in the UC-Ca group compared with the UC-NonCa group ( $p=0.018$ ) (Fig. 2). Mean Log<sub>2</sub>-normalized mRNA expression level for

UC-Ca group and UC-NonCa group was -0.35 and 0.6, respectively.

**Methylation status of *RUNX3* gene promoter region.** There was no significant difference in the methylation status of the *RUNX3* gene promoter region, with neither group showing hypermethylation (Table II).

**DNA copy number of *RUNX3*.** The mean copy number of *RUNX3* was significantly greater in the UC-NonCa group compared with the UC-Ca group (3.04 vs. 1.99,  $p=0.0016$ ) (Fig. 3).

**mRNA expression level and DNA copy number of *RUNX3*.** mRNA expression levels were then examined according to DNA copy number. The standard deviation of the *RUNX3* copy number of non-neoplastic tissue adjacent to sporadic CRC tumours was 0.1 (data not shown), so we determined the cut-off value for copy number gain as 2.2 ( $2.0 + 2 \times \text{SD}$ ). Samples were divided into a high copy number ( $>2.2$ ) or low copy number ( $\leq 2.2$ ) group. The *RUNX3* mRNA level was significantly higher in the high copy number group compared

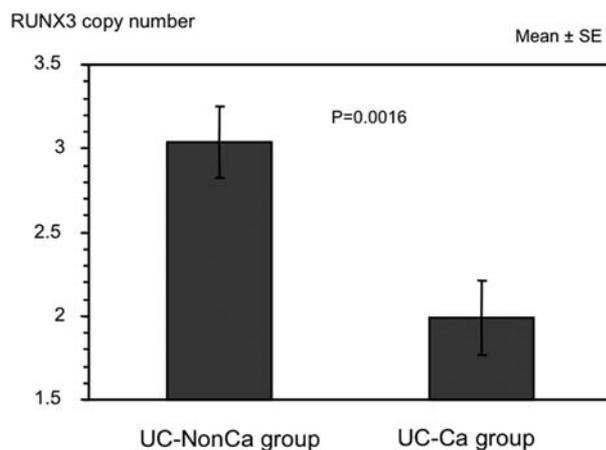


Figure 3. *RUNX3* DNA copy number. UC-NonCa group showed significantly higher *RUNX3* DNA copy number than UC-Ca group (3.04 vs. 1.99,  $p=0.0016$ ).

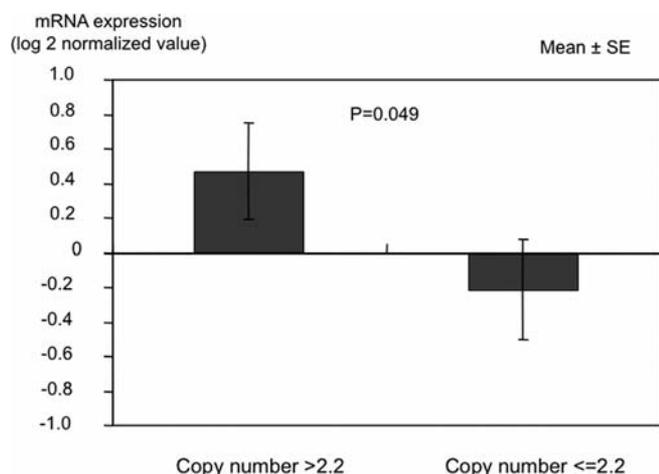


Figure 4. *RUNX3* mRNA expression level and DNA copy number. High copy number group (>2.2) showed significantly higher mRNA expression level than low copy number group ( $\leq 2.2$ ) ( $p=0.049$ ).

to the low copy number group ( $p=0.049$ ) (Fig. 4). Mean Log<sub>2</sub>-normalized mRNA expression level for the high copy number group and the low copy number group was 0.47 and -0.21, respectively.

**Prediction of UC-associated neoplasm development by *RUNX3* mRNA level and DNA copy number.** ROC analysis against UC-associated neoplasms revealed the AUC to be 0.74 and the copy number to be 0.85 (Fig. 5). To maximize sensitivity and specificity, we set the cut-off points as 0.027 and 2.06 for mRNA and DNA copy number, respectively. Using mRNA expression data, we were able to predict the development of UC-associated neoplasms with an accuracy of 74.3%. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 76.5%, 72.2%, 72.2% and 76.5%, respectively. There were nine misclassifiers: five in the UC-Ca group and four in the UC-NonCa group. Using

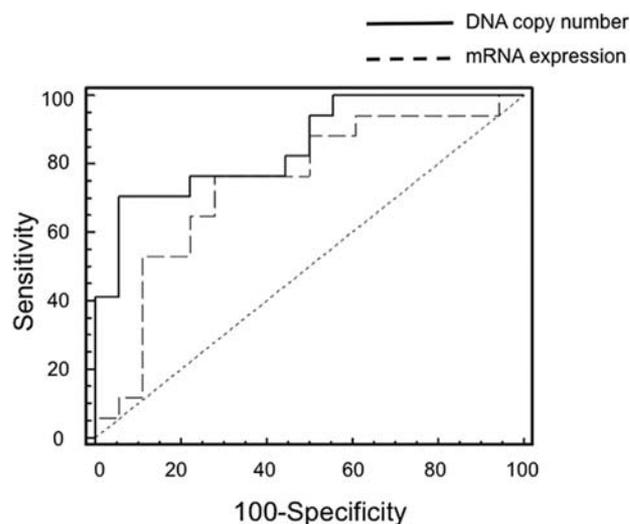


Figure 5. ROC curve for UC-Cancer prediction model based on *RUNX3* mRNA expression and DNA copy number. Predicted probability of UC-associated cancer computed from logistic regression models including *RUNX3* mRNA and DNA copy number. Specificity and sensitivity computed at each possible cut-off on predicted probability for two models. AUC values compared for two models.

DNA copy number data, we were able to predict the development of UC-associated neoplasms with an accuracy of 82.9%. The sensitivity, specificity, PPV and NPV were 70.6%, 94.4%, 92.3% and 77.3%, respectively. There were six misclassifiers: one in the UC-Ca group and five in the UC-NonCa group.

## Discussion

In the current analysis of *RUNX3* expression in non-neoplastic rectal mucosa of UC patients, we were able to show that non-UC-Ca patients have significantly higher *RUNX3* mRNA expression levels than UC-Ca patients. Increased DNA copy numbers of *RUNX3* are a plausible mechanism for this change. Based on the DNA copy number, we were able to predict the development of UC-associated neoplasms with an accuracy of 82.9%. The present model may enable more efficacious surveillance for UC patients by selecting high risk UC-Ca patients.

In UC-carcinogenesis, it is suggested that there is a field effect of chronic inflammation with a need for constantly increased re-epithelialisation and the suppression of cell cycle-regulating proteins, which may make genes susceptible to genetic damage. Indeed, previous studies have shown that specific gene alteration is already present in the non-neoplastic mucosa of UC-CA patients (5-8). However, to date, *RUNX3* has not been identified as one of these genes. This is the first study to show altered *RUNX3* expression levels in UC-Ca patients. *RUNX3* expression is decreased in various cancers, (10-12) and was significantly decreased in UC-Ca patients compared with UC-NonCa patients in the present study. From these results, it is difficult to conclude that there is a causal relationship between *RUNX3* expression levels and the development of UC-Ca because there is no concrete evidence to support this hypothesis. However, considering that *RUNX3*

is a tumor suppressor gene, decreased expression of *RUNX3* may help to increase the risk of developing UC-Ca in long-standing UC patients.

Next, we examined the mechanisms of increased *RUNX3* expression in UC-NonCa patients. Previous studies have shown that hypermethylation plays an important role in controlling *RUNX3* mRNA expression levels in various cancers including gastric cancer (13-19). Therefore, we first examined the hypermethylation of CpG islands in the promoter region of *RUNX3*. However, we failed to show any relation between hypermethylation status and *RUNX3* expression level because not a single case in either UC-Ca or UC-NonCa group showed the hypermethylation in *RUNX3* promoter DNA.

Recently, DNA copy number has gained attention as a possible mechanism for altering gene expression in various diseases (27,28). Copy number variations may be either inherited or caused by *de novo* mutations and can influence gene expression (28,29). In inflammatory bowel disease, changes in the DNA copy number have been reported in Crohn's disease. Fellermann *et al* reported that changes in the human  $\beta$ -defensin 2 gene copy number at the  $\beta$ -defensin locus predisposes individuals to Crohn's disease, most likely through diminished  $\beta$ -defensin expression (27). We therefore focused on DNA copy number as a possible regulator of *RUNX3* mRNA expression and found that the UC-NonCa group had both higher copy numbers and *RUNX3* expression levels than the UC-Ca group. These results suggest that increased expression of *RUNX3* mRNA in UC-NonCa patients might be attributable to the increased *RUNX3* DNA copy number. It is not known when the copy number changes occurred in UC-NonCa patients, but considering that the risk of UC-Ca increases with the duration of disease, we speculate that copy number changes might be acquired as *de novo* mutations during the long period of disease duration.

An important clinical implication of the present study is the prediction of patients at a higher risk of developing UC-Ca. In predicting the development of UC-associated neoplasm, we used mRNA expression level and DNA copy number. Although ROC analysis showed a higher AUC for DNA copy number (0.85) than mRNA expression level (0.74), the difference did not reach statistical significance and seemed to be attributable to a comparatively small number of cases. In fact, the overall predictive accuracy was higher by DNA copy number (82.9%) than mRNA expression level (74.3%), suggesting that *RUNX3* DNA copy number is a more efficacious predictive marker than mRNA expression level. The accuracy level in the present model was not as high as our previous DNA microarray study, which showed a 98.1% accuracy rate, but required 40 genes to achieve this prediction accuracy. On the other hand, the present study only assessed *RUNX3* DNA copy number, so is a more cost-effective method and is easier to apply in clinical settings.

One limitation of the present study is that because of a comparatively small number of patients, we could not validate the accuracy of the predictive model. To further confirm its accuracy, the *RUNX3* DNA copy number should be validated by testing at another institution, preferably with a prospective study of a larger number of patients. The second point is that *RUNX3* expression levels are influenced by the degree of

inflammation (20) which cannot be excluded from influencing the current results. However, in the present study, UC patients with active inflammation were excluded from analysis. Finally, the precise mechanism of action of *RUNX3* has not been fully clarified, and analysis of this would greatly contribute to our understanding of the mechanisms of UC-Ca.

In conclusion, patients without UC-Ca showed higher expression of *RUNX3* than those with UC-Ca. We show that this increase could have been caused by an increase in *RUNX3* DNA copy number. Furthermore, using the *RUNX3* copy number, we were able to predict patients with and without UC-Ca with an accuracy of 80%, and suggest that this predictive model may be useful in detecting patients at high risk of developing UC-Ca, thereby increasing the efficacy of surveillance colonoscopy for UC patients.

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