N-myc downstream-regulated gene 1 as a downregulated target gene of PTEN in the controlling of tumourigenesis in endometrioid carcinoma

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Background & objectives: Mutation/deletion of PTEN has been known to be involved in the development of many cancers including endometrial carcinoma. NDRG1 (N-myc downstream-regulated gene 1) is reported to be associated with tumourigenesis. PTEN expression has been shown to be correlated with NDRG1 in both prostate and breast cancer. In this study, we explored the possibility that PTEN alteration may cause carcinogenesis of endometrioid carcinoma by regulating the expression of the NDRG1 gene.

Methods: Tissue blocks of 103 patients with pathologically confirmed endometrioid carcinoma were included. All the carcinoma tissues were accompanied with varied degree of necrosis. Using two-step method and avidin-biotin peroxidase complex immunohistochemistry method, the correlation of the two genes expression in ischaemic area and the relationship of NDRG1 expression between ischaemic and non-ischaemic area in endometrioid carcinomas was evaluated.

Results: PTEN alteration and NDRG1 expressions were significantly increased in the ischaemic area of endometrioid carcinoma compared with their expressions in the normal endometrium respectively ($P<0.001$, $P<0.001$). A positive correlation was found between PTEN alteration and NDRG1 expression in the ischaemic area of endometrioid carcinoma.

Interpretation & conclusions: We suggest that NDRG1 may be an important candidate gene in facilitating endometrium carcinogenesis in the adaptation of hypoxia for survival. Alteration of PTEN may upregulate NDRG1 expression, which plays an important role in the process leading to endometrial carcinogenesis.

Key words Endometrioid carcinoma - NDRG1 - PTEN

Endometrial carcinoma is the most diagnosed cancer in the female genital tract. Each year, about 1,42,000 women are diagnosed with this disease and 42,000 women die from it. Carcinogenesis is implicated in destruction of balance between oncogene and anti-oncogene. Mutation of the oncogene or inactivation of the anti-oncogene can lead to cell transformation.

In the first place, a well known tumour suppressor gene, PTEN (phosphatase and tensin homologue deleted
on chromosome 10), also called MMAC1 (mutated in multiple advanced cancers), is a kind of house-keeping gene involved in many organs to be accounting for a major role in the process of cellular growth and differentiation of many tissues. Its major function relies on its phosphatase activity and subsequent antagonism of the phosphatidylinositol 3 kinase (PI3K)/AKT pathway. Several groups\(^1\) have found that PTEN loss/mutation led to accumulation of phosphatidylinositol tri phosphate (PIP3) and activation of AKT/protein kinase B (PKB) pathway in endometrial carcinogenesis and progression. As a serine/threonine protein kinase, AKT exerts its function by phosphorylating key intermediate signaling molecules and further regulates many cancer cells’ survival.

On the other hand, hypoxic regions within solid tumours are often accompanied with a more malignant tumour phenotype and unfavourable prognosis. Oxygen-deprived cells alter genes expression to obtain blood supply and survive. Some researchers demonstrated that under hypoxic state, HIF-1 (hypoxia-inducible transcription factor-1) is upregulated at transcriptional level by expression of activated Akt/PKB\(^5\) due to loss/mutation of PTEN\(^6\)-11. As a pivotal transcription factor, HIF-1 plays a major role in tumour progression by activating a number of genes critically involved in adaptation to hypoxia, such as NDRG1\(^12\)-17, VEGF\(^8\),\(^9\),\(^17\), and EPO\(^13\), which are called hypoxia-inducible genes.

NDRG1 (N-Myc downstream regulated gene 1) is a popular gene which has evoked many interests throughout the world in recent years. However, its exact mechanism has not been well elucidated yet. It is a downstream target gene of N-myc and comprises one of four NDRG family members. Many groups have demonstrated that nickel compounds can mimic hypoxia to induce HIF-1 transactivation and further augment NDRG1 protein expression\(^13\),\(^15\)-19. A computer analysis has found that there is one HIF-1 binding site in the promoter and two HIF-1 binding sites in the 3’ untranslated region of NDRG1\(^18\). Using mouse epidermal Cl41 cells exposure to nickel compounds, Li et al\(^7\) suggested that NDRG1 was a gene adjusted by PI-3K/Akt/PKB through HIF-1 pathway, which was closely associated with tumourgenesis and development.

Given that (i) loss/mutation of PTEN or hypoxia can activate PI-3K-Akt pathway, (ii) as a second messenger, PI-3K increases the expression of HIF-1 transcription factor, (iii) HIF-1 proteins combine with HIF-1 binding sites in the promoter of NDRG1 and induce its transcription and translation, and (iv) NDRG1 then induces cell’s transformation to facilitate the cell’s survival, we hypothesized that PTEN loss/mutation may lead to endometrial carcinogenesis through upregulation of NDRG1 in response to hypoxia.

In the study, we tested if PTEN loss/mutation leads to upregulation of NDRG1 expression during carcinogenesis of endometrioid carcinoma.

Material & Methods

Tissue specimens: Tissue blocks were selected from a retrospective cohort of 103 patients with pathologically confirmed endometrioid carcinoma (type I endometrial carcinoma), from 2003 to 2005 in the Department of Pathology, The first people’s Hospital in Shanghai, China. The age of the patients varied from 32 to 81 yr. The patients were classified into two groups according to the age, \(a<50\) yr old group and a \(\geq50\) yr old group.

Only those tissues accompanied with varied degree of necrosis were selected. These were classified into three groups according to the WHO classification 2003\(^20\), consisting of 35 cases of grade I, 38 cases of grade II and 30 cases of grade III. Forty cases of normal endometrium were selected for controls from women undergoing gynaecological procedures for benign conditions (20 proliferative and 20 secretory phase, respectively). The study was approved by the institutional ethics committee.

Immunohistochemistry: Consecutive paraffin sections of 4 \(\mu\)m thickness from each block were mounted onto silanized slides and dried overnight at 37°C followed by 2 h at 60°C. The sections were deparaffinized in xylene and rehydrated in a graded series of alcohol solutions.

Two-step method was used for PTEN according to the manufacturer’s instructions. (DakoCytomation Envision System Labelled Polymer-HRP Anti-Mouse, DAKO, Glostrup, Denmark). Immunostaining was performed using the mouse monoclonal primary antibody PTEN (Cat. No. M-0665. Antibody Diagnostica Inc, USA), 1:60, for 60 min, followed by a monoclonal secondary antibody (DakoCytomation Envision System Labelled Polymer-HRP Anti-Mouse, DAKO, Glostrup, Denmark). The sections were visualized with the EnVision Detection Kit (DAKO, Glostrup, Denmark) using diaminobenzidine chromogen as substrate and counterstained with haematoxylin. Controls for the immunostaining were performed using normal endometrial tissue control
sections and positive normal cell compartments (i.e., stroma) within test sections.

Avidin-biotin peroxidase complex (ABC) method was performed for NDRG1 according to goat ABC Staining System (sc-2023, Santa Cruz, USA) according to the manufacturer’s instructions. Slides were incubated for 10 min in 0.1-1 per cent hydrogen peroxide diluted in PBS to quench endogenous peroxidase activity, for one hour in 1.5 per cent blocking serum in PBS and incubated with primary antibody NDRG1 (N-19;sc-19464), goat polyclonal primary antibody, Santa Cruz, USA), 1:80, overnight at 4°C. Then sections were incubated for 30 min with biotinylated secondary antibody at approximately 1 µg/ml, 30 min with AB enzyme reagent and 20-30 min with peroxidase substrate. The sections were with counterstained haematoxylin for 5-10 sec.

The study was carried out in the Immunohistochemical unit of our department.

All the immunostaining slides and the corresponding HE slides were evaluated independently by two expert pathologists who were blinded to the patients’ clinical data.

Analysis of immunohistochemical staining: Firstly, HE slides of the selected specimens were reviewed, marked for the necrotic area and confirmed the perinecrotic zone as ischaemic area according to the previous study 21.

Normally, PTEN expression was located in the nuclear region. Using stromal PTEN signal as positive control, immunohistochemical PTEN expression in glands around necrotic regions was classified as PTEN expressing or PTEN null. Those specimens that contained any PTEN-null gland in the ischaemic area were regarded as PTEN loss/mutation positive. Of the 103 cases, 65 were PTEN loss/mutation positive in the ischaemic area of the endometrioid carcinoma (Fig. 1A, Fig. 2A and Fig. 3A). The positive rate (63.11%) of PTEN loss/mutation in the ischaemic area of endometrioid carcinoma was markedly higher than that (15%) in the normal endometrium (Table I, \(P<0.001\)).

NDRG1 was scored positive as long as there were brown granules in the glandular cytoplasm. In the ischaemic area of the endometrioid carcinoma, NDRG1 was positive in 82 cases with a expression rate of 79.61 per cent (Fig. 1B, Fig. 2B and Fig. 3B). In the non-

### Results

**PTEN loss/mutation and NDRG1 expression in normal endometrium:** PTEN loss/mutation and NDRG1 expression were respectively examined in 40 cases of normal endometrium. PTEN loss/mutation was positive in six cases and negative in 34. NDRG1 was negative in most of the normal endometrium (35/40) with only 5 cases expressed weakly.

**PTEN loss/mutation and NDRG1 expression were significantly increased in ischaemic area of endometrioid carcinoma:** Those specimens that contained any PTEN-null gland in the ischaemic area were regarded as PTEN loss/mutation positive. Of the 103 cases, 65 were PTEN loss/mutation positive in the ischaemic area of the endometrioid carcinoma (Fig. 1A, Fig. 2A and Fig. 3A). The positive rate (63.11%) of PTEN loss/mutation in the ischaemic area of endometrioid carcinoma was markedly higher than that (15%) in the normal endometrium (Table I, \(P<0.001\)).

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<table>
<thead>
<tr>
<th>endometrium</th>
<th>N</th>
<th>PTEN loss/mutation</th>
<th>NDRG1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal endometrium</td>
<td>40</td>
<td>6</td>
<td>34</td>
</tr>
<tr>
<td>Ischaemic area of endometrioid carcinoma</td>
<td>103</td>
<td>65*</td>
<td>38</td>
</tr>
</tbody>
</table>

*\(P<0.001\) compared to normal (Fisher’s exact test)
Fig. 1. Comparison between PTEN loss/mutation (A) and NDRG1 (B) expression in the same part adjacent to the necrotic foci in the endometrioid carcinoma. Normally PTEN expression was located in the nuclear region. Using stromal PTEN signal as positive control, Fig. 1A illustrated typical examples of PTEN loss/mutation in the carcinoma glands. Fig. 1B showed that NDRG1 highly expressed in the cytoplasm of the carcinoma glands in the counterpart. The arrowheads indicate the necrotic areas.

Fig. 2. Comparison between PTEN loss/mutation (A) and NDRG1 (B) expression in the same part near the necrotic regions in the endometrioid carcinoma. PTEN immunoreactivity was only found in the nuclear part of the stromal cells without expression in the carcinoma glands in Fig. 2A. However, in Fig. 2B, high immunoreactivity of NDRG1 was found in the cytoplasm of the carcinoma glands without expression in the stromal cells. The arrowheads indicate the necrotic areas.

Table II. Comparison of immunoreactive intensity of NDRG1 between ischaemic area and non-ischaemic area on 103 paired measurements.

<table>
<thead>
<tr>
<th>Ischaemic area</th>
<th>N</th>
<th>non-ischaemic area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative or weakly positive (60)</td>
</tr>
<tr>
<td>Negative or weakly positive</td>
<td>53</td>
<td>39</td>
</tr>
<tr>
<td>Highly positive</td>
<td>50</td>
<td>21</td>
</tr>
</tbody>
</table>

In the ischaemic area, NDRG1 positive immunostaining was seen in 70 of 103 with an expression rate of 67.96 per cent. Its positive rate in the ischaemic area of endometrioid carcinoma was significantly higher than that (12.5%) in the normal endometrium (Table I, P<0.001).

NDRG1 protein expression was not significantly higher in ischaemic area than in non-ischaemic area in endometrioid carcinoma: According to the immunoreactive intensity of NDRG1, 82 positive cases in the ischaemic area and 70 in the non-ischaemic area were respectively classified into three levels as weak (+), moderate (++) and high (+++). Since the weak (+) cases had only few positive cells, the negative (-) and the weak (+) cases were combined to form one group, while the moderate (++)

Table III. Correlation between PTEN loss/mutation and NDRG1 in ischaemic area

<table>
<thead>
<tr>
<th>PTEN loss/mutation</th>
<th>N</th>
<th>NDRG1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>65</td>
<td>57</td>
</tr>
<tr>
<td>Negative</td>
<td>38</td>
<td>25</td>
</tr>
</tbody>
</table>

P<0.01 (McNemar test)
Fig. 3. Comparison of PTEN loss/mutation (A) and NDRG1 (B) between normal glands and carcinoma glands in the same part around the necrotic regions in the endometrioid carcinoma. Fig. 3A showed that the positive signals of PTEN located in the nuclear of the peripheral normal glands (left upper and left below), whereas no signals were shown in the central carcinoma glands. It was found in Fig. 3B that NDRG1 highly expressed in the cytoplasm of the central carcinoma glands while negatively expressing in the peripheral normal glands. This indicated the inversed expression between the two genes.

<table>
<thead>
<tr>
<th>Age</th>
<th>N</th>
<th>PTEN loss/mutation</th>
<th>NDRG1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive (65)</td>
<td>Negative (38)</td>
</tr>
<tr>
<td>&lt;50 yr</td>
<td>18</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>≥50 yr</td>
<td>85</td>
<td>55</td>
<td>30</td>
</tr>
</tbody>
</table>

and the high (++) cases were combined to form another group.

As shown in Table II, in the ischaemic area, 53 cases were negative or weakly positive for NDRG1 and 50 were highly positive. In the non-ischaemic area, 60 cases were negative or weakly positive and 43 cases were highly positive; 39 cases were negative or weakly positive both in the ischaemic area and in the non-ischaemic area, while 29 cases were highly positive both in the ischaemic area and in the non-ischaemic area. A comparison of immunoreactive intensity of NDRG1 between the ischaemic area and the non-ischaemic area was made by Mc Nemar test. The result suggested that NDRG1 expression in the ischaemic area was not different from that in the non-ischaemic area.

PTEN loss/mutation was positively correlated with NDRG1 expression in ischaemic area of endometrioid carcinoma: By using stromal signals as control, PTEN loss/mutation was found in the nuclear region of the carcinoma glands, whereas NDRG1 was highly expressed in the cytoplasm of the counterparts (Fig. 1 and Fig. 2). As shown in Fig. 3, PTEN loss/mutation was detected in the central carcinoma glands by using signals of the peripheral normal glands as control, whereas NDRG1 highly expressed in the cytoplasm of the central carcinoma glands with negative expression in the peripheral normal glands. This indicated an inversed expression between PTEN and NDRG1 in the same part.

Simultaneously positive number of PTEN loss/mutation and NDRG1 was 57 cases, and the simultaneously negative number was 13 cases. The correlation between PTEN loss/mutation and NDRG1 expression was assessed by Mc Nemar test. The results suggested that PTEN loss/mutation was significantly related to the expression of NDRG1 in the ischaemic area of endometrioid carcinoma (P<0.01, Table III).

PTEN loss/mutation in endometrioid carcinoma was not related to patients’ age: PTEN loss/mutation was positive in 10 of 18 cases of <50 yr old group and negative in cases. It was positive in 55 of 85 cases of ≥50 yr old group and negative in 30 cases. The loss/mutation of PTEN was not related to the patients’ age in the endometrioid carcinoma (Table IV).

NDRG1 expression in endometrioid carcinoma was not related to patients’ age: NDRG1 was positive in 13 of 18 cases of <50 yr old group and negative in 5 cases. It was positive in 69 of 85 cases of ≥50 yr old and negative in 16 cases. The expression of NDRG1 was not related
to the patients’ age in the endometrioid carcinoma (Table IV).

**Discussion**

There is now accumulating evidence that NDRG1 is closely related to tumourigenesis, but its role in the endometrioid carcinoma remains unclear. Bandyopadhyay et al. revealed that PTEN could regulate the expression of NDRG1 gene in prostate and breast cancer. However, the relationship between the two genes in the endometrioid carcinoma has not been elucidated yet. In the present study, we investigated whether NDRG1 was involved in cell’s transformation to facilitate the cell’s survival in response to hypoxia in endometrial carcinogenesis and whether NDRG1 exerting its carcinogenetic mechanism was regulated by PTEN loss/mutation.

Firstly, we detected the expression of PTEN loss/mutation and NDRG1 in 40 cases of normal endometrium. Only 5 cases of NDRG1 were found weakly immunoreactive, and only 6 cases of PTEN loss/mutation were detected in the normal endometrium.

Secondly, we investigated PTEN loss/mutation in the ischaemic area of endometrioid carcinoma. Our data showed that PTEN loss/mutation was increased in the ischaemic area of the endometrioid carcinoma comparing with a low loss/mutation rate in the normal endometrium. These results were consistent with the previous reports that PTEN loss/mutation was closely related to carcinogenesis in endometrial carcinoma. However, the rate of PTEN loss/mutation (63.11%) in our study was relatively higher than that in earlier reports. The reason might be that the selected tissue blocks were all accompanied with varied degree of necrosis. While the ischaemic areas adjacent to necrotic foci were confirmed to be the hypoxia areas, it seemed that hypoxia might be a factor to increase the loss/mutation of PTEN.

Thirdly, we demonstrated that NDRG1 expression was significantly increased in the endometrioid carcinoma comparing with that in the normal endometrium. It raises the possibility that NDRG1 is involved in the endometrioid carcinogenesis. We further found that NDRG1 protein expression in the ischaemic area was higher than that in the non-ischaemic area, although no statistical difference was found. As a target gene of HIF-1, NDRG1 may implicate in facilitating endometrium carcinogenesis in the adaptation of hypoxia for survival.

Since higher expressions of PTEN alteration and NDRG1 were found in the ischaemic area of the endometrioid carcinoma than that in the normal endometrium, we explored the correlation between PTEN loss/mutation and NDRG1 expression in the ischaemic area in the endometrioid carcinoma. We found an inversed expression between PTEN and NDRG1 in the same part. The analysis indicated that PTEN loss/mutation was positively correlated with NDRG1 expression in the ischaemic area of the endometrioid carcinoma.

It has been documented that NDRG1 was a target gene of HIF-1, which was upregulated under conditions of cellular stress, such as DNA damage and severe hypoxia. Maybe this is a possible molecular mechanism which can explain the higher expression of NDRG1 in the ischaemic area in response to hypoxia. HIF-1 is a target of the PI-3K/Akt signaling pathway which could be controlled by PTEN gene loss/mutation through increasing the PIP3 level under hypoxic conditions.

Based on earlier reports, we speculated that PTEN loss/mutation may result in enhanced NDRG1 expression through Akt/PKB signaling pathways, and subsequent HIF-1 mediated transcription in the development of endometrioid carcinoma. The elucidation of the role that PTEN loss/mutation can upregulate NDRG1 in the development of the endometrioid carcinoma may serve as a theoretical basis for prevention and treatment of this kind of cancer, and perhaps, also other PTEN-null human cancers in the future.

However, this is only a preliminary study on the relationship between PTEN loss/mutation and NDRG1 at the protein level. The underlying mechanism of endometrial carcinogenesis is not clear yet, thus the question whether there is an absolute association between PTEN loss/mutation and NDRG1 cannot be answered at present. Further studies need to be done to answer these questions.

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**References**


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