Original Article
The homing and distribution of protection to bone marrow by using multidrug resistance gene 1 in laryngocarcinoma mice with intensified chemotherapy

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Abstract: Objective: The aim of this study is to observe the homing and distribution of the foreign multidrug resistance gene 1 (mdr1) in gene therapy. Method: After the bone marrow mononuclear cells of male BABL/C which was transferred with retrovirus-mediated mdr1 were transplanted into female Babl/c which was conducted radiotherapy with 60Co-γ, the expression and distribution of foreign mdr1 in the bone marrow mononuclear cells, peripheral blood mononuclear cells and chief organs were detected by using immunohistochemistry, reverse transcriptase polymerase chain reaction (RT-PCR) and Fluorescence in Situ Hybridization (FISH), meanwhile, the homing of the bone marrow was monitored by immunohistochemistry and FISH. Result: There was no expression of external mdr1 in chief organs and tumor, P-glucoprotein expression percentage of the bone marrow mononuclear cells, peripheral blood mononuclear cells and tumor were respectively (9.58±1.24)%,(8.31±1.62)% and (48.52±11.42)% before performing chemotherapy, the homing rate of the bone marrow transferred with retrovirus-mediated mdr1 is 67.2%. Conclusion: The expression of the external mdr1 in bone marrow mononuclear cells and peripheral blood mononuclear cells was high, but no expression in the chief organs and tumors, the homing rate of the transplant of bone marrow transferred with retrovirus-mediated mdr1 was significantly increased.

Keywords: Multidrug resistance, gene therapy, laryngocarcinoma, distribution

Introduction
As the main attributes for the failure of tumor chemotherapy, multidrug resistance, and bone marrow suppression have aroused wide attentions [1]. The mdr1 transfected into the bone marrow mononuclear cells can protect the bone marrow suppression in the chemotherapy and thus improve the tumor killing effect in the intensified chemotherapy [2, 3]. This study transplanted the bone marrow hematopoietic cells of male Babl/c which transfected with mdr1 into female Babl/c and detected expressions of mdr1 in bone marrow hematopoietic cells, peripheral blood mononuclear cells, and chief organs. On this basis, it investigated the protection effect of external mdr1 to bone marrow and the homing of mdr1 in bone marrow after transplantation.

Materials and methods
Packaging cells and materials
PA317-HaMDR1/A virus packaging cells were constructed by Tianjin Blood Institute, Chinese Academy of Sciences and reserved in the laboratory of the present study; mouse anti human P-gp monoclonal antibody and S-P ultra sensitive immunohistochemical kit were produced by Fuzhou Maixin Biological Technology Development Company; RT-PCR Kit (AMV) Ver.3.0 was produced by Takara Biotechnology Co., Ltd.; clean Balb/c inbred mouse of 6-8 weeks old and (20±2) g in weight(donor: female mice, receptor: male mice) were provided by the Animal experimental center of Hangzhou Normal University; Hep-2 cell lines of laryngeal squamous cell carcinoma were reserved in the laboratory of the present study; cisplatin was produced by Qilu Pharmaceutical Co. Ltd; Y chromosome probe labeled by Cy-3 was produced by Cambio Company.

Test methods
The concentrated virus supernatant collected, the PA317-HaMDR1/A virus packaging cells produced by transfection of bone marrow mononuclear cells (BM-MNC), 5% CO₂, and the iscove’s modified dulbecco’s medium (IMDM)
with 10% calf serum were cultured in the CO₂ incubator at 37°C. Supernatant was collected for every 2-3 days and then centrifuged at 4000 r/min for 2 h. After the supernatant was discarded, the residuals were made into concentrated virus supernatant [2]. The virus titer was measured as 4.12×10⁶ cfu/ml. Under aseptic conditions, BM-MNCs were separated from mouse bone marrow molecular cells that were isolated from the femurs of the lower limbs of mouse using lymphocyte separation liquid (specific gravity 1.077) and density gradient centrifugation method. By 5×10⁵ cells and 1 ml for each well, the BM-MNCs were then moistly cultured in 24 well plates with 0.4 ml IMDM culture liquid (20% fetal bovine serum) ml, 0.4 ml concentrated virus supernatant, 10 μl 3% L-glutamine (3 μg), 5 μl 2-mercapto ethanol (10 mol/L), 0.1 ml 10% BSA, 7.5 μl Polyberene (6 μg), 20 μl rmlL-3 (50 μg), 20 μl rmlL-6 (50 μg), 10 μl rmFit-3 (20 μg), 20 μl rmSCF (50 μg) in each well. Half of the medium was changed for every two days. After 4 days, the bone marrow transplantation and transfection effect were determined.

Determination of mdr1 transfection effect: (1) The integration of external human mdr1 in BM-MNC was firstly detected using RT-PCR method at the genetic level. Then according to the kit instructions, the total RNA was extracted from the pre-tested transfected cells. The PCR reaction was described as that the external human mdr1 was degenerated for 4 min at 95°C, degenerated for 30 s at 94°C, annealed for 45 s at 58°C, and extended for 1 min at 72°C in turn. This process was repeated for 35 cycles. Afterwards, the human mdr1 were preserved at 72°C for 7 min; concerning premiers, the sense and antisense of mdr1 (157 bp) were 5’-CCCATCATTGCA ATA GCA GG-3’ and 5’-GTT CAA ACTTCT GCTCCTCA-3’ respectively, while those of β-actin (527 bp) were 5’-CTACAATGA-GCTGC GTGTTG-3’ and 5’-AAGGAAGGCTGGAAAG-AGTGCC-3’ respectively. The product was analyzed by 1.5% agarose gel electrophoresis. (2) According to the instructions of kit, the transfection rate was detected using immunohistochemistry at protein level. Each specimen was provided with 5 smears. For each smear, we randomly selected 200 cells from 5 views and calculated the proportion of the positive cells at each level. The modeling and grouping of tumor: the cell lines of Hep-2 laryngeal squamous cell carcinoma were cultured in 5% CO₂ incubators at 37°C. The concentration of the cell lines was firstly adjusted to optimum at good growth condition of the cell lines and then diluted to be 1×10⁷ after the cell lines were rinsed by PBS for 3 times. The cell line solutions obtained were then injected beneath the skin of the left axilla of mouse, with 0.1 ml for each mouse. One day later, the mice were irradiated by 10⁶ Co-γ ray at intensity of 1.5 Gy. After irradiation, they were transplanted with the BM-MNCs of the same mouse splice that were transfected or untransfected with mdr1 via tail vein at the 48th and 72th hour, 1×10⁶ BM-MNCs for each mouse, and 0.2 ml of each time. A total of 32 Balb/c mice were randomly divided into four groups (8 mice for each group), including control group A (the mice only pretreated by 10⁶ Co-γ ray irradiation), blank control group B (the tumor-bearing mice treated by chemotherapy of normal saline infusion in equal amount), negative control group C (the tumor-bearing mice treated by chemotherapy of procedurally transplanting with the hematopoietic cells untransfected by mdr1 genes), transfection group D (the tumor-bearing mice treated by intensified chemotherapy of procedurally transplanting with the hematopoietic cells transfected by mdr1 genes).

Chemotherapy: The cisplatin applied is 2.5 mg/kg (X) in conventional chemotherapy, while the dose is gradually increased for 1.5X, 2.0X, 3.0X, 4.0X, and 5.0X in intensified chemotherapy. The cisplatin was intraperitoneally injected into the mice for once a week.

Determination of the in vivo distribution of the transfected mdr1: In normal way, the BM-MNCs were extracted from mice and the integration condition of the external mdr1 were tested by RT-PCR method. Then the colonization rates after the transplantation the BM-MNCs transfected with mdr1 genes were detected using dropping immunohistochemistry. Subsequently, the MNCs of peripheral blood were isolated using density centrifugation and related P-gp expressions were detected using dropping immunohistochemistry. Then the mdr1 expressions in heart, liver, spleen, kidney, lung, and tumor tissues were measured using RT-PCR. Finally, the distributions of the mdr1 genes were explored using immunohistochemistry.

The homing and in vivo distribution of mdr1 detected by FISH: The drops of BM-MNCs iso-
lated from the femurs and tibias of the lower limbs of female mice were detected using Y chromosome probe. Then the homing rates of 250 BM-MNCs were calculated. The in vivo distribution in the sections of heart, liver, spleen, kidney, lung, and tumor tissues were inspected using Y chromosome probe: (1) Pretreatment: the sections were baked in the oven at 60°C for 6-8 h; dewaxing: the sections were placed in xylene in 3 flasks for 10-20 min in turn; the slides were then oscillated for 5 min in 100% ethanol water-bath for twice. After removed out and dried in air, they were placed in the 2×SSC water bath of 75°C for about 5-20 min and then digested at 45°C for 10-25 min using 0.25 mg/ml proteinase K. Afterwards, the slides were oscillated for 5 min in 2×SSC water bath at room temperature; Subsequently, they were placed in the 70%, 85%, and 100% gradient ethanol for 2 min in sequence and finally dried in air. (2) Hybridization: we removed the probes and corresponding hybridization buffer solution from the refrigerator at -20°C. After centrifugation, 1 ul probe was diluted into 10 ul in a 0.5 ml EP tube using the buffer solution. After uniformly mixed, the probe solutions were centrifuged for 2-3 seconds. We took 10 ul mixed liquid onto the slides with preset position. The slides were then covered by 22×22 mm cover glasses. After air bubbles were removed, the surrounding of the cover glasses were sealed using glues. The slides were then melted in hybrid instrument at 72°C for 10 min. Then they were placed in the moist hybrid box, which was finally put into the incubator at 37°C in dark overnight. (3) Washing the slides: we took the slides from the hybrid box in the incubator and carefully removed the sealing glues and cover glasses. Then the slides were oscillated for 2 min in a flake containing 0.4×SSC and preheated at water bath of 75°C for 30 min (error ≤ ±1°C). Next, they were oscillated for 1 min in a flake containing 2×SSC/0.1% NP-40. After the liquids were adsorbed using absorbent paper, they were dried in dark. The dry slides were added with 10 ul DAPI in the hybrid zone and then covered with 24×50 mm cover glass. (4) Finally, they were observed by microscope after the optical filters were adjusted by color observation.

Statistical method

SPSS18.0 statistical software was employed in the analysis. The test data, which were represented by mean±standard deviation, were conducted with normal test and homogeneity of variance test. The χ² test and variance analysis results were listed in from of line×column.

Results

Detection of in vitro transfection effect of mdr1 genes

Four days after transfection, the total RNA of the BM-MNCs were extracted and amplified by RT-PCR. The amplified total RNA was detected with a specific amplification band integrated at 157 bp. Thus it is confirmed that the mdr1 had been successfully transfected into the BM-MNCs. P-gp immunohistochemistry suggested that the mouse BM-MNCs transfected with concentrated virus supernatant was (31.24±3.76)%. The positive expressions mainly distributed in the membranes of BM-MNCs, while negative expressions were not observed in the control group.

Table 1. Expression of P-gp in mouse bone marrow mononuclear cell and peripheral blood mononuclear cells after chemotherapy

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<tr>
<td>Blood</td>
<td>11.25±1.23</td>
<td>14.83±1.65</td>
<td>17.36±1.47</td>
<td>18.54±1.63</td>
<td>21.21±1.60</td>
<td>23.48±1.37</td>
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<tr>
<td>Bone</td>
<td>9.73±1.64</td>
<td>10.54±1.54</td>
<td>11.32±1.21</td>
<td>12.64±1.72</td>
<td>14.42±1.36</td>
<td>18.63±1.84</td>
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* vs * ′P<0.05, ′ vs ′P<0.05, ′ vs ′P>0.05.

**Figure 1.** Positive expression of P-gp in tumor of control group (group A) (SP×400).
Expression of mdr1 gene in peripheral bloods and bone marrows of receptor mice

Before chemotherapy, the positive P-gp expression rate of the MNCs in bone marrow and peripheral blood achieved (9.58±1.24)% and (8.31±1.62)% respectively. With the intensifying of chemotherapy, the test results showed that the P-gp expression yield of mdr1 kept stable and higher expressions in the bone marrow hematopoietic cells from the first week of the transfection. With the dose increasing of drugs in chemotherapy, the positive P-gp expression rate gradually increased. Moreover, the positive P-gp expression rates in the peripheral blood and bone marrow were presented statistically significant differences in the sixth week and first week respectively (t=7.52, 6.32, P<0.05); the P-gp expressions in peripheral blood were higher than those in bone marrow. But the difference show no statistical significance (t=2.957, P>0.05) (as shown in Table 1).

Expression of mdrl in the chief organs and tumor tissues of receptor mice

We separated the heart, liver, spleen, kidney, lung, and tumor tissues from the mice transfected with mdrl and isolated the total RNA. RT-PCR detection suggested that the RNA showed no specific bands of mdrl expressions after amplification. Immunohistochemistry detection also failed to find the P-gp expressions in the organs. However, P-gp expression was found to be high in the tumor tissues. In the tumor tissues of Group A (normal control group without chemotherapy), the positive P-gp expression rate was (48.52±11.42)% (Figure 1). Variance analysis on each group implied that the four groups exhibited no statistically significant differences during the first 3 weeks (F=97.538, P<0.000). The multiple comparisons among groups yielded the following results: the positive P-gp expression rates of Group C and Group D showed no statistically significant difference with that of Group B respectively, P>0.05; In the fourth week, the positive P-gp expression rate of Group C showed statistically significant difference with that of Group D, P>0.05; the positive P-gp expression rate of Group D in the sixth week showed statistical significance with that in the 1st week, P<0.05 (as shown in Table 2).

Protection of mdr1 on the haematopoietic function of bone marrow

Since the tumor-bearing mice were pretreated by radiation before the first transplantation, the leukocytes were not on a normal level in the chemotherapy. Before chemotherapy, there was no significant difference on the number of leukocytes in peripheral blood of each group (F=2.136, P>0.05). This result suggested that the leukocyte numbers in the peripheral blood of the tumor-bearing mice in each group were at the same level and allowed for chemotherapy. After intensified chemotherapy, the leukocyte numbers in the peripheral blood of Group D and Group B showed statistical significance in each time point (t=6.532, P<0.01) (as shown in Table 3); 15 days after the first procedural transplantation, Group D and Group C were also of statistical significance in aspects of the leukocyte numbers in peripheral blood (t=7.316, P<0.01). Compared with the Group B, the leukocyte number reduction were slower in Group C.

FISH detection of the homing and in vivo distribution of BM-MNCs transfected by mdrl genes

The BM-MNCs isolated from the lower limb femurs and tibias showed fluorescent expressions of Y chromosome. In the 250 cells selected, there were 168 positive cells, with a homing rate of 168/250=67.2% (Figure 2). Fluorescent expressions of Y chromosome were not detected in tumor tissue, heart, liver, spleen, kidney, lung, brain, and intestine.

Table 2. Positive expression of P-gp in tumor after chemotherapy (%)

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<tbody>
<tr>
<td>Group A</td>
<td>48.52±11.42</td>
<td>47.34±10.31</td>
<td>46.78±10.54</td>
<td>48.67±9.92</td>
<td>46.45±10.33</td>
<td>47.91±8.38</td>
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<tr>
<td>Group B</td>
<td>48.34±9.35</td>
<td>51.21±10.47</td>
<td>54.65±10.07</td>
<td>54.89±8.43</td>
<td>55.65±10.56</td>
<td>56.65±9.32</td>
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<tr>
<td>Group C</td>
<td>46.13±8.62</td>
<td>50.39±10.72</td>
<td>51.17±8.36</td>
<td>51.76±9.51</td>
<td>52.37±8.75</td>
<td>52.95±9.66</td>
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<tr>
<td>Group D</td>
<td>44.76±10.57a</td>
<td>47.15±7.25</td>
<td>50.21±10.38</td>
<td>52.37±11.24</td>
<td>55.91±8.86</td>
<td>59.63±9.57b</td>
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*a vs b P<0.05.
Table 3. Leukocyte numbers in the peripheral blood in tumor-bearing group after chemotherapy (×10^9/L)

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<th>9 d</th>
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<th>15 d</th>
<th>18 d</th>
<th>21 d</th>
<th>28 d</th>
<th>35 d</th>
<th>42 d</th>
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<tr>
<td>Group B</td>
<td>6.91±0.46</td>
<td>3.73±0.16</td>
<td>3.32±0.12</td>
<td>2.43±0.51</td>
<td>2.12±0.44</td>
<td>1.63±0.56</td>
<td>1.36±0.51</td>
<td>0.88±0.32</td>
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<tr>
<td>Group C</td>
<td>6.78±0.45</td>
<td>6.74±0.51a</td>
<td>4.45±0.42</td>
<td>3.93±0.63</td>
<td>3.76±0.35</td>
<td>2.72±0.81</td>
<td>2.53±0.32</td>
<td>1.65±0.47</td>
<td>1.04±0.35</td>
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<tr>
<td>Group D</td>
<td>6.64±0.39</td>
<td>6.52±0.34a</td>
<td>5.67±0.82a</td>
<td>5.53±0.54a</td>
<td>5.14±0.62a</td>
<td>4.84±0.63a</td>
<td>4.37±0.32a</td>
<td>3.97±0.53a</td>
<td>3.32±0.26a</td>
<td>2.56±0.34</td>
<td>2.14±0.37</td>
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Compared with the Group B *P<0.01, Compared with the Group C *P<0.01, "-" show mice have died.
Discussions

As an effective method in resisting the bone marrow suppression for drug, the stability and security of mdr1 therapy were attached with increasing importance gradually [4, 5]. This study found that mdr1 showed effective expressions in hematopoietic cells of bone marrow after transfected into the transplant of hematopoietic cells. Meanwhile, the mice in the test kept a high leukocyte number in the peripheral blood, which made it greatly possible for the improvement of drug dose and protection of bone marrow. The radiotherapy before the bone marrow transplantation was targeted at vacating the “niche” in the receptors. Moreover, as for the allogeneic bone marrow transplantation, it reduced the graft-versus-host reactions of transplants caused by the entering of allogeneic T lymphocytes [6, 7]. Niche refers to the places for the proliferation and differentiation of hematopoietic stem/progenitor cells in the hematopoietic microenvironment. It is also an adjustment region constituted by hematopoietic stromal cells and multiple locally enriched hematopoietic regulation factors [8]. It is located in the blood sinus and is vacated by the dead stem cells in the pretreatment [9]. Due to the very low differentiation rate, hematopoietic stem cell’s reproductive death follows with finite times of differentiation in a few days of irradiation injury [10-15]. Therefore, it is speculated that the niche vacation process is completed 3-4 days after irradiation. After BM-MNCS transplantation, the implantation of stem cells in bone marrow is a highly specific active process, which is called as homing [16]. The homing process is generally completed in 12-36 hours after the transplantation. FISH detection revealed that, in the first week of the transplantation of the BM-MNCS transfected by mdr1, the homing rate achieved 61.6%. This result confirmed that a larger number of transplanted hematopoietic cells can realize homing. However, immunohistochemical staining showed that, in the first week, the positive P-gp expression rate was (9.73±1.64)% in the bone marrow. It is speculated that this result may be related to in vitro transfection efficiency. Stem cells moved between peripheral blood and bone marrow in form of mobilization. After chemotherapy, it was measured that the positive P-gp expression rate achieved (11.25±1.23)% in the MNS of peripheral blood in the first week. In addition, with the intensifying of chemotherapy, the positive P-gp expression rate increased gradually possibly attributing to that, in short time period, the intensified chemotherapy induces the P-gp expressions of BM-MNCS and killed the BM-MNCS with lower P-gp expressions.

The human mdr1 and mouse mdr1 are highly homological. Their difference lies in the first transmembrane region on the amino terminal and carboxyl terminal. Additionally, human adr1 is also similar with the protein gene transported by bacteria. Therefore, mdr gene is a highly conserved gene family [15, 17, 18]. In the present study, with the intensifying of chemotherapy, tumor tissues showed significantly up-regulated positive P-gp expressions. However, the differences of the positive P-gp expressions among groups were not statistically significant. This results indicated that the mdr1 gene expressions in tumor tissue were unaffected by the transplantation of the BM-MNCs transfected by external mdr1 gene. RT-PCR detection also implied that there was no external mdr1 gene in the tumor tissues. Thus it can be confirmed that drugs up-regulates the expressions of mdr1 gene and thus leading to the acquired drug resistance. In addition, Y chromosome fluorescence expressions were also not found in the tumor tissue, heart, liver, spleen, kidney, lung, brain, and intestine of mice. P-gp expressions were detected in the tumor tissues of mice using immunohistochemistry. The outcome suggested there were internal mdr1 in the laryngeal carcinoma of mice. The higher primary drug resistance of the internal mdr1 may be one of the reasons for unsatisfied laryngeal carcinoma chemotherapy effect. Meanwhile, it fur-

Figure 2. Fluorescence positive expression of Y-chromosome in bone MNC received transplantation of the transfected cells (FISH×400).
ther proved that the external mdr1 transfected were not localized to the tumor tissue and chief organs of mice.

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Disclosure of conflict of interest

None.

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