Effect of Retinoic Acid and Chemotherapeutic Agents on Ultrastructural Localization of Myc-N in Neuroblastoma

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Summary

The effect mechanism of pharmacological agents used in neuroblastoma treatment on myc-N expression is still unclear. Myc-N amplification does not change with any agent. The aim of this study is to investigate the effect of chemotherapeutic agents and retinoic acid on ultra structural localization of myc-N in neuroblastoma. We analyzed ultra structural localization changes of myc-N by immunoelectron microscopy in myc-N positive, Kelly human neuroblastoma cell line using retinoic acid and cytotoxic drugs (cisplatin, vincristine, cyclophosphamide, etoposide, doxorubicin) and their combinations incubated for 24 hours in preoptimised LD50 doses in cell culture compared with control conditions. Myc-N was applied by immunoelectron microscopy method using colloidal gold for visualisation. Results were scored semi quantitatively as negative, mild, moderate, or high positive in nucleus, ribosomal and cell membrane. Immunogold particles labeling myc-N was high in nucleus, ribosomes and low in cell membrane in control tumor cells without any drug. It was moderate in nucleus in retinoic acid, cyclophosphamide, etoposide, cisplatin and their combinations groups. The nuclear expression was mild in, vincristine, doxorubicin and their combinations groups. It was negative in ribosomes in all combination groups and doxorubicin and retinoic acid combined with vincristine group. Chemotherapeutic agents and their combinations caused a prominent decrease in myc-N expression in cell membrane, a medium level decrease in ribosomal level and a low decrease in nuclear ultra structural localization. Myc-N expression is reduced with cytotoxic agents and retinoic acid especially in ribosomal units. Retinoic acid combined with vincristine or doxorubicin is the most effective combination to reduce myc-N expression.

Key words: Neuroblastoma, Myc-N, Ultrastructural Localization

Retinoik Asit ve Kemoterapötik Ajanların Nöroblastomda Nmyc'in Ultrasrtürüktürel Lokalizasyonuna Etkisi

Özet


Anahtar Kelimeler: Nöroblastom, Myc-N, Ultrastrüktürel Lokalizasyon

INTRODUCTION

Neuroblastoma (NB) is an important pediatric tumor that myc-N amplification is a well known poor prognostic indicator(2,22,23,25). It is derived from primordial neural crest and it is the most common extra cranial solid tumor. This pediatric tumor has diverse biological characteristics and clinical behaviors(17,18,21). Stage 3 and 4 neuroblastoma cases still have worse prognosis although there is an improve in prognosis. Recurrence and metastasis are still common in NB(6).

The tumor development in NB is dependent on defects in several genes such as Loss of Heterozygosity in 1p, 2q, 9p,11q,14q(9). However myc-N amplification is the most important genetic alteration among all. Myc-N is amplified in one third of NB cases(15). It is included in risk categorization of NB. Myc-N amplification is associated with worse overall survival, and disease free survival(24). The myc genes presumably use the same mechanisms to transform cells. Myc has been studied as therapeutic target in many aspects (antisense DNA-oligonucleotides, RNA-interference, and antisense mRNA)(12). It is a difficult target since its localization, genetic properties and etc. The current used therapeutic agents might also effect the expression of myc-N. The effect and mechanism of pharmacological agents used in neuroblastoma treatment on myc-N expression is still unclear. Immunohistochemical expression of myc-N protein changes after chemotherapeutic applications although Myc-N amplification does not change with any agent that was tested in our previous studies(1). Immunoelectron microscopic applications give possibility to investigate ultrastructural localizations of proteins by labeling with immunogold particles that are visible in electron microscopy(8). The effect of retinoic acid and chemotherapeutic agents on ultra structural changes of myc-N in neuroblastoma is not studied yet. The aim of this study is to investigate the effect of chemotherapeutic agents and retinoic acid on ultra structural localization of myc-N in neuroblastoma.

MATERIAL AND METHODS

In this study, we analyzed ultra structural localization changes of myc-N by immunoelectron microscopy in myc-N positive, Kelly human neuroblastoma cell line using retinoic acid and cytotoxic drugs.

Cell Culture: (cisplatin, vincristine, cyclophosphamide, etoposide, doksorubicin) and their combinations incubated for 24 hours in preoptimised LD50 doses in cell culture compared with control conditions as described before(1).

Immuoelectron Microscopy: Cells that were collected by cell scraper were immediately fixed in gluteraldehyde fixative and stored at +4 C° for minimum
24 hours. After centrifugation at 2000G, each cell pellet was mixed with 0.5 cc melted 2% agar-agar (40°C) on a slide to have collected material for immunoelectron microscopic procedures and myc-N was applied by immunoelectron microscopy method using colloidal gold for visualization. After cooling the solidified agar with cells were cut to small pieces (2x2 mm). The pieces each in an eppendorf were dehydrated in increasing ethanol (50, 70, 90%, 15 minutes each. They were put in polymerized resin gradually (1:1 ethanol: resin for 2h. twice then 1:2 ethanol:resin for 2 h. twice and resin overnight, change resin, embed in capsule with resin and polymerize at 55°C for 24 h. ). After control by semi thin sections, ultrathin (<0.1 µm) sections were mounted on grids. Then immunogold staining was done as described by Haase et al.(13). The Primary was applied for 2 h (N-Myc (2), Santacruz, sc-142, antihuman, mouse monoclonal) at 1:100 dilutions. The secondary goat antimouse colloidal gold coupled antibody (AlexaFluor 488 10nm goat anti-mouse IgG conjugate) was applied at 1:50 dilution for 1h. After proper washings double-contrasting was done by 2% uranyl acetate and 1% lead citrate incubation.

**Analysis:** Results were scored semi quantitatively as negative, mild, moderate, or high positive in nuclear, ribosomal and cell membrane ultra structural localizations.

**RESULTS**

Immunogold particles labeling myc-N was high in nucleus, ribosomes and low in cell membrane in control tumor cells without any drug. It was moderate in nucleus in retinoic acid, cyclophosphamide, etoposide, cisplatin and their combinations groups. The nuclear expression was mild in, vincristine, doxorubicin and their combinations groups. N-myc expression was negative in cell membrane in all drugs. It was negative in ribosomes in all combination groups and doxorubicin and retinoic acid combined with vincristine group. Immunoelectron microscopic results showed that chemotherapeutic agents and their combinations caused a prominent decrease in myc-N expression in cell membrane, a medium level decrease in ribosomal level and a low decrease in nuclear ultrastructural localization (Table 1) (Figure 1).

**Table 1:** Immunoelectron microscopic results of chemotherapeutic agents and their combinations.

<table>
<thead>
<tr>
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<th>Nucleer</th>
<th>Ribosomal</th>
<th>Membranous</th>
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<tbody>
<tr>
<td>Control</td>
<td>+++</td>
<td>++/++++</td>
<td>+</td>
</tr>
<tr>
<td>Retinoic Acid (RA)</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vincristine</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Cisplatin</td>
<td>++</td>
<td>++</td>
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<td>Endoxan</td>
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<tr>
<td>Doxorubicin</td>
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<tr>
<td>Etoposide</td>
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<td>++</td>
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<tr>
<td>RA+Vincristin</td>
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<tr>
<td>RA+Cisplatin</td>
<td>++</td>
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<tr>
<td>RA+Endoxan</td>
<td>++</td>
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<tr>
<td>RA+doxorubicin</td>
<td>+</td>
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<tr>
<td>RA+etoposide</td>
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DISCUSSION

Our results showing chemotherapeutic agents and their combinations cause as a prominent decrease in myc-N expression in cell membrane, a medium level decrease in ribosomal level and a low decrease in nuclear ultrastructural localization indicate that the synthesis of protein is decreased although the amplified gene is active after the drugs. In our previous study we showed that amplification does not change.

As a member of Myc proto-oncogene family, Myc-N promotes proliferation. Its amplification and/or expression play an important role in aggressiveness in NB\(^5\). Myc-N overexpression has been shown to promote cell cycle progression and to increase DNA synthesis in an inducible system. Myc-N amplification is known to cause DNA instability. MycN might be overexpressed in MycN amplification negative cases as a worse prognostic factor\(^{15}\). Myc protein acts by binding to transcription repressor Max. This causes inappropriate activation of growth promoting genes\(^5\). Subsequent formation of a Myc-Max protein heterodimer in the tumor cell nucleus prevents cellular differentiation\(^{10}\). Retinoic acid as a differentiating agent decreases myc_N protein level as shown in this study especially in ribosomal localization. Survival patient studies show that stratification therapy with high dose chemotherapy and stem cell transplantation improved survival\(^{4,19,23}\). That is why Myc-N is not an independent prognostic factor in multivariate analysis. The improvement in the treatment approach does not indicate that myc-N has lost importance as a prognostic factor\(^{13}\).

Myc RNA localizes mainly to nucleolus. Its morphologic manifestation is thought to be enlargement of nucleoli in undifferentiated NB. Mitotic karyorrhectic index also is thought to be one of the morphologic manifestations of Myc N amplification\(^{13}\). Myc-N overexpression has been shown to shorten the G1 phase of the NB cell cycle and induce the reentry of quillicent cells into the cell cycle\(^3\).

Electron microscopic in situ hybridization is also very useful to determine the precise sub cellular site of expression of very low levels of mRNA\(^{20}\). Our study is the first time that mycN protein is shown to be

Figure 1: Immunogold labeled Myc-N expression. Arrow head: membrane, short arrow: nuclear, long arrow: ribosomal.
observed rather than nuclear compartment and it's changed by chemotherapeutic agents. The ribosomal compartment is the protein production center of the cell. Retinoic acid in combination with cisplatin changes the mycN expression to negative in ribosomal and as well as membranous compartment. That is why this combination might be effective in control of minimal residual disease since vincristine or doxorubicin is not advised to be given continuously. However, their combinations also decrease nuclear expression more than cisplatin and retinoic acid.

Retinoic acid induces differentiation of NB cells in vitro and Myc-N undergoes transcriptionally mediated downregulation. Regulation of Myc-N expression occurs at multiple levels, including gene transcription, premature termination of translation and translocation\(^{(7,14,16,26,27,28)}\). Our study supports Myc-N expression decrease by showing the proteins sub cellular localization and amount changes.

In conclusion Myc-N expression is reduced with cytotoxic agents and retinoic acid in neuroblastoma especially in ribosomal units. Retinoic acid combined with vincristine or doxorubicin is the most effective combination to reduce myc-N expression. We showed that these agents affect myc-N function.

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