Molecular Aspects during Multi-step Chemical Induced Carcinogenesis in the Lung and Pancreas

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Abstract: Alterations of various genes during N-nitrosobis(2-hydroxypropyl)amine (BHP) -induced lung carcinogenesis in rats and N-nitrosobis(2-oxopropyl)amine (BOP) -induced pancreatic duct carcinogenesis in hamsters were summarized. In both carcinogenesis, Ki-ras mutation was the early event in lung alveolar and pancreatic duct hyperplasias. Although p53 mutation was not detected in lung adenocarcinomas, it was revealed as late events in transplantable pancreatic carcinoma and its cell lines. FHIT alterations were found in rat lung adenocarcinomas and in hamster pancreatic duct carcinomas. The induced lesions in both rat lung and hamster pancreas were histologically and genetically similar to those seen in humans. It was evident from this study that various gene alterations were accumulated from preneoplastic lesions to carcinomas induced by nitrosoamines in rat lung and hamster pancreas. Since cancer is a disease of gene alterations, the detection of genetic and environmental factors which induce organ specific gene alterations is important for the prevention and early detection and treatment of lung and pancreatic cancers.

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Key words: lung carcinogenesis, pancreatic carcinogenesis, nitrosamine, rat, hamster, BHP, BOP

Introduction

It is generally accepted that carcinogenesis is multi-step consisting of qualitatively different stages, such as initiation, promotion, and progression¹. To analyze molecular events in different stages of carcinogenesis using animal models provides important informations for the prevention, detection and treatment of human cancers.

Lung cancer is the first and adenocarcinoma of the pancreatic ducts is the fifth leading cause of cancer death in both the United States and Japan in 2000²,³. The early detection and prevention offer the only hope for effective treatment of those deadly diseases. However, rate-limiting molecular events for the development of lung and pancreatic cancers are still largely obscure.

Previously, we established a model for the development of non-small cell lung cancers (NSCLCs) in rats given N-nitrosobis(2-hydroxypropyl)amine (BHP) in drinking water, with high yields of adenocarcinomas⁴,⁵. Because the step-by-step development of lung cancers is accessible with this model, molecular mechanisms involved at each step can be readily investigated. We also established a rapid production model for pancreatic duct adenocarcinoma in hamsters by N-nitrosobis(2-oxopropyl)amine (BOP)⁶ which is a BHP-related compound.

Here, we report the genetic alterations during BHP induced lung and BOP induced pancreatic duct carcinogenesis in rats and hamsters, respectively, which we have investigated for more than a decade⁴,⁵.

Genetic Alterations on Lung Carcinogenesis Induced by BHP in Rats

Male Wistar rats (Japan SLC Inc., Shizuoka, Japan) received BHP (Nakalai Tesque Co. Ltd., Kyoto, Japan) at a concentration of 2000 ppm in drinking water for 12 weeks and then drinking water without BHP. The experimental protocol is schematically shown in Fig. 1.

To obtain normal lung tissues the same strain of rats...
were maintained free from carcinogen exposure throughout the experimental period. All rats were sacrificed by exsanguination from the abdominal aorta under light ether anesthesia, and the lung was immediately excised and grossly apparent tumors were dissected from surrounding tissue. Samples were frozen in liquid nitrogen and stored at –80°C until analysis. Portions of the tumors were also fixed in 10% neutrally buffered formalin at 4°C, routinely processed for embedding in paraffin, sectioned, and stained with hematoxylin and eosin for histological examination.

For mutational analysis, polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and reverse transcription (RT)-PCR-SSCP analysis were performed7–13. For the investigation of aberrant transcription and gene expression, RT-PCR, northern blot analysis and reverse transcription (RT)-PCR-SSCP analysis and ribonuclease protection assay (RPA) were carried out9–17. Western blot analysis was also used for assessment of protein expression9,12. The genes and the specific methods used for the analysis of the alterations in rats are summarized in Table 1.

The gene alterations during step-by-step lung carcinogenesis induced by BHP in rats are summarized in Fig. 2. Histologically, adenocarcinoma developed through sequential changes of alveolar hyperplasia to adenoma. The gene alterations during step-by-step pancreatic carcinogenesis induced by BOP in hamsters are summarized in Table 1. The alterations and immunohistochemistry 21,26,27 were performed. The genes and the specific methods used for the analysis of the alterations in hamsters are summarized in Table 1.

The gene alterations during step-by-step pancreatic duct carcinogenesis induced by BOP in hamsters are summarized in Fig. 4. Histologically, adenocarcinoma developed through sequential changes of duct epithelium from hyperplasia, atypical hyperplasia, intraductal

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**Table 1. Methods Used for Molecular Analysis**

<table>
<thead>
<tr>
<th>Methods</th>
<th>Alterations</th>
<th>Genes and Proteins</th>
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<tr>
<td>PCR-SSCP</td>
<td>mutation</td>
<td>Ki-ras&lt;7,20&gt;, β-catenin&lt;8,21&gt;, APC&lt;8&gt;, p53&lt;7,22&gt;, Ha-ras&lt;7&gt;, FHIT&lt;9&gt;</td>
</tr>
<tr>
<td>RT-PCR-SSCP</td>
<td>mutation</td>
<td>Smad2&lt;11&gt;, Smad4&lt;11&gt;, TGFβRII&lt;10&gt;, M6P/IGF2R&lt;13&gt;, RB2/p130&lt;12&gt;</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>expression</td>
<td>TGFβRII&lt;10&gt;, TGFα&lt;23&gt;, VEGF&lt;23&gt;, FHT&lt;10,24&gt;, M6P/IGF2R&lt;13&gt;</td>
</tr>
<tr>
<td>RPA</td>
<td>expression</td>
<td>TGFβ&lt;16&gt;, Interleukins&lt;17&gt;</td>
</tr>
<tr>
<td>Southern blot</td>
<td>shortening</td>
<td>Telomerase&lt;25&gt;</td>
</tr>
<tr>
<td>Northern blot</td>
<td>expression</td>
<td>VEGF&lt;15&gt;, VEGFRs&lt;15&gt;, midkine&lt;14,27&gt;, RB2/p130&lt;12&gt;, COX-2&lt;*&gt;, MMPs&lt;26&gt;, MT-MMP&lt;26&gt;, TIMPs&lt;26&gt;, nm23&lt;23,28&gt;, c-jun&lt;28&gt;, bax&lt;29, bcl2&lt;29&gt;</td>
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<td>Western blot</td>
<td>expression</td>
<td>FHIT&lt;9&gt;, pRB2/p130&lt;12&gt;</td>
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<td>TRAP assay</td>
<td>activation</td>
<td>Telomerase&lt;25&gt;</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>expression</td>
<td>MMP-2&lt;26&gt;, β-catenin&lt;21&gt;, midkine&lt;27&gt;</td>
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Parentheses of ( ) references No. were used in rats, and of < > reference No. used in hamsters. <*>: unpublished data.


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**Genetic Alterations on Pancreatic Carcinogenesis Induced by BOP in Hamsters**

A rapid production model incorporating the principle of selection by resistance to cytotoxicity demonstrated earlier for liver carcinogenesis in rats<18,19> was established for pancreatic carcinoma development in Syrian hamsters (Japan SLCL Inc., Shizuoka, Japan). The basic protocol is shown in Fig. 3. BOP at doses of 50 mg/kg body weight were given subcutaneously as the initiation, followed by three cycles of augmentation pressure. Eleven days after BOP initiation, hamsters received four daily i.p. injection of 500 mg/kg ethionine while being maintained on a choline-deficient (CD) diet. At the end of the CD diet, 800 mg/kg methionine was given by i.p. injection. The animals were returned to the basal diet and given 20 mg BOP/kg thereafter. Augmentation pressure was repeated beginning on day 26 and again on day 40, and the hamsters were killed 70 days after the beginning of the experiment. The whole pancreas was carefully dissected and ductal lesions confirmed by light microscope were carefully microdissected for molecular analysis.

In hamsters, the methods of PCR-SSCP<20–22>, RT-PCR<23,24>, Southern blot<25>, northern blot<26,27> TRAP assay<25>, and immunohistochemistry<21,26,27> were performed. The genes and the specific methods used for the analysis of the alterations in hamsters are summarized in Table 1.

The gene alterations during step-by-step pancreatic duct carcinogenesis induced by BOP in hamsters are summarized in Fig. 4. Histologically, adenocarcinoma developed through sequential changes of duct epithelium from hyperplasia, atypical hyperplasia, intraductal...
carcinoma to invasive carcinoma. As DNA alters, Ki-ras mutation was detected from the hyperplasia to invasive carcinoma, and p53 mutation was detected in transplantable tumors and cell lines. The expressions of mRNA for midkine, nm23, matrix metalloproteinase-2 (MMP-2), membrane-type MMP (MT-MMP), tissue inhibitor of metalloproteinase-2 (TIMP-2), and cyclooxygenase (COX)-2 were overexpressed in invasive carcinomas. The activity of telomerase was also increased in invasive carcinomas.

**Molecular Aspects during Lung and Pancreatic Carcinogenesis**

The present studies were performed in two different models in rats and hamsters given nitrosamines. The nitrosamines of BHP and BOP are metabolically related compounds and BOP possess the strongest carcinogenic activity among BHP metabolites. It has been reported that the main target organ of BHP is the lung but not the pancreas in rats and that of BOP, subcutaneous injections, is the pancreas but not the lung in hamsters. Induced adenomatous lesions in the lung and pancreas are histologically similar to the lesions seen in humans, suggesting those two models are very useful for studying multistep carcinogenesis and for the implication to humans.

In this study, Ki-ras mutations were early event in both lung and pancreatic carcinogenesis. In human lung cancers, Ki-ras mutations have been detected in NSCLCs with a higher frequency in adenocarcinomas than in squamous cell carcinomas. Our previous studies in rat lung carcinogenesis showed also a higher frequency of Ki-ras mutation (72%) in adenocarcinomas while 50% mutation in squamous cell carcinomas. Therefore, Ki-ras mutations are playing one of the important roles in lung carcinogenesis both in animal model and human. The high frequency of Ki-ras mutation in human pancreatic duct adenocarcinomas is well known. Furthermore, human pancreatic adenomas are in line with findings presented by Yanagisawa et al., who suggested the possible existence of an adenoma-carcinoma sequence, with Ki-ras mutation as a possible important event in this phase. Again, Ki-ras mutation of G to A transition in the second position of codon 12 play the important roles in pancreatic duct carcinogenesis but environmental factors including chemicals which induce Ki-ras mutations are totally unknown.

p53 mutations have been detected in preneoplastic lesions in the lung of human, suggesting that they occur in the early stage of lung carcinogenesis. In contrast, it has
been reported that no or infrequent p53 mutations were found in BHP induced rat lung carcinogenesis. Therefore, the roles of p53 mutations in lung carcinogenesis are still obscure but an etiological factor for lung cancer might be involved for p53 mutations. In contrast, p53 mutations were detected in the transplantable carcinoma and their cell lines both of which were established from hamster pancreatic duct carcinomas induced by BOP and in human pancreatic duct carcinomas. These results suggest that p53 mutations are involved in the progression stage of pancreatic carcinogenesis.

FHIT alterations have been found in BHP induced rat lung adenocarcinomas and in BOP induced hamster pancreatic duct carcinomas. A high frequency of FHIT alterations has been reported in human lung cancers. Recent report described that the FHIT gene is postulated to be a target of environmental carcinogenesis causing human cancer, particularly tobacco smoking and associated nitrosamines in lung cancers. The present results of FHIT alterations in lung and pancreatic duct carcinomas might be caused by nitrosamine of BHP and BOP, respectively.

In human lung and pancreatic cancers, there were few mutations of β-catenin and APC genes. In animal models, we detected frequent alterations of β-catenin and APC in BHP induced rat lung carcinogenesis, but no mutation of β-catenin observed in BOP induced hamster pancreatic carcinomas. Therefore, the molecular pathways underlying human lung cancers and BHP induced rat lung tumors appear to be quite different. In contrast, the mutations of β-catenin and APC may not be involved in the development of BOP induced hamster pancreatic carcinomas, even in human.

Alterations of TGFβ signaling pathway-associated genes, such as TGFβRII, Smad2, Smad4, and M6P/IGF2R, have been reported in several human cancers, including lung and pancreas, these alterations resulting in resistance of cancer cells to the effects of TGFβ inhibition. In BHP induced rat lung adenocarcinomas, we found mutations of TGFβRII, Smad2, Smad4, reduced expression of TGFβRII, and aberrant transcripts of M6P/IGF2R. Therefore, the disturbance of TGFβ signaling pathway may play important roles in the development of lung adenocarcinomas. Now we are also investigating the involvement of TGFβ signaling pathway in BOP induced hamster pancreatic duct carcinomas.

The over-expressions of various genes were found in BHP induced rat lung adenocarcinomas and BOP induced hamster pancreatic duct carcinomas. It is evident that multistep carcinogenesis is accompanied with cellular and DNA events and various gene alterations were accumulated in cancer cells. Some gene alterations, for examples, MMP-2, TGF-α and VEGF give the informations for the development of new chemotherapeutic agents as one of the markers. Studies to clarify the organ specific gene alterations for cancer development which contribute to the
prevention, detection, and treatment are further necessary.

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