Distinct allelic expression patterns of imprinted IGF2 in adenocarcinoma and squamous cell carcinoma of the lung

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Received January 14, 2014; Accepted August 22, 2014

DOI: 10.3892/ol.2014.2572

Abstract. The insulin-like growth factor 2 gene (IGF2) is an imprinting gene, which mediates cell growth and apoptosis. The loss of imprinting (LOI) of IGF2 has been associated with the development of cancer. In the present study, loss LOI of IGF2 in lung cancer was analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in combination with DNA sequencing of samples collected by laser capture microdissection. The status of each sample was assigned as imprinting when PCR-RFLP revealed only one band or sequence with a single peak; otherwise, the case was classified as LOI. LOI was identified in eight out of 13 adenocarcinoma cases (62%), but was not detected in any of the nine squamous cell carcinoma cases (0%). These results suggest that IGF2 LOI is involved in the molecular pathogenesis of lung adenocarcinoma, but not squamous cell carcinoma, and that LOI may be detected through increased IGF2 expression levels.

Introduction

Insulin-like growth factor 2 (IGF2) is an important mediator of cell growth and apoptosis that is paternally expressed. IGF2 loss of imprinting (LOI) is predominantly indicated by the activation of the usually silenced maternal allele, with subsequent expression of the two gene copies (1). This is associated with a number of hereditary overgrowth conditions, including Beckwith-Wiedemann syndrome (2) and cancer that exhibits cell overgrowth resulting from IGF2 overexpression (3). LOI has been reported in a variety of cancer types, in particular colorectal carcinoma (4-6). However, to the best of our knowledge, only one study examining LOI in lung carcinoma (7) has been published; LOI was detected in 47% adenocarcinomas, although the prevalence of LOI in squamous cell carcinomas was not mentioned. However, other studies have found the prevalence of LOI in squamous cell carcinomas and urothelial carcinomas in other organs to be relatively low. For example, the prevalence was found to be 21% in esophageal cancer (8) and 22% in bladder cancer (9). By contrast, the prevalence of LOI was 44-54% (4,7) and 49% (10) in colorectal adenocarcinoma and gastric carcinoma, respectively. However, considerable variation has been reported.

Personalized cancer therapy has been applied to lung adenocarcinoma patients through the development of molecular-targeted therapeutic drugs against driver oncogenes. For example, lung adenocarcinoma patients with an epidermal growth factor receptor (EGFR) mutation or with an ELM4-ALK fusion protein have been shown to respond well to the corresponding drugs (11). Similarly, molecular-targeted therapy for insulin growth factors (IGFs) has been developed for a variety of cancer types, including non-small cell lung cancer (12,13). Therapeutic methods that target the IGFI receptor (IGF1R) have achieved certain success, although a modified therapy that targets and insulin receptor (IR) has proved more effective (13). IGFI and IR are receptors for IGF2 that induce signal transduction resulting in cell growth; however, the IGF2 receptor interrupts IGF2 signal induction (13). Silencing of the IGF2 gene was recently reported to result in apoptosis only for IGF2 LOI colorectal carcinomas (14). Thus, IGF2 LOI lung carcinoma appears to be a good candidate for molecular-targeted therapy.

To examine IGF2 LOI, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) has been previously employed, but precise analysis is hampered by a number of problems, including lymphocyte contamination and heteroduplex formation during PCR (15). In the present study, the precise incidence of IGF2 LOI in lung carcinomas was examined using PCR-RFLP in combination with DNA sequencing of samples obtained by a laser capture microdissection (LCM) method, as reported previously (16).
**Materials and methods**

**Materials.** Tissue samples were obtained from 32 patients with lung cancer (19 with adenocarcinoma, 12 with squamous cell carcinoma and one with large cell carcinoma). Sections of carcinoma tissues and non-tumor lung tissue were removed and frozen immediately following surgery at Kanazawa University Cancer Research Hospital (Kanazawa, Japan). The tissues were stored at -80°C until further analysis.

**LCM.** Frozen 10-μm tissue sections were fixed with 70% ethanol for 10 min and stained with Kernechtrot (Merck, Darmstadt, Germany). A total of ~1,000 carcinoma cells in the frozen sections were punched out using a LCM system (LM100; Olympus Corporation, Tokyo, Japan) and collected on a plastic cap (CapSure™ LCM Caps; Arcturus, Mountain View, CA, USA). All specimens were assessed histologically by a pathologist (Professor E. Kawahara).

**PCR-RFLP and direct sequencing.** Genomic DNA from the frozen sections of the normal tissues was extracted using a Wizard® SV Genomic DNA Purification system (Promega Corporation, Fitchburg, WI, USA). To extract total RNA from the microdissected carcinoma cells, a PicoPure™ RNA isolation kit (Arcturus) was used. The extracted RNA was further purified using the acid guanidinium phenol chloroform method, and possible contaminating DNAs were digested with DNase I (Takara, Tokyo, Japan). Aliquots of 1 µl extracted total RNA in a volume of 20 µl were converted to cDNA using AMV reverse transcriptase (Promega Corporation). The reverse transcription reaction was performed for 30 min at 42°C and then the sample was heated for 5 min at 99°C to inactivate the enzyme. Genomic DNA from normal whole tissues was amplified by PCR using the following primer pair for exon 9 of human IGF2: Forward, 5'-CTTGGACTTGAGTCAAATTGG-3' and reverse, 5'-GGTCGTGCCAATTACATTTCA-3'. The tumor cDNAs obtained from the microdissected carcinoma cells. The PCR products from the carcinoma cells revealed either two bands or a single band following Apal analysis, and either two C and T peaks or one peak with a possible background peak subsequent to sequence analysis (Fig. 1). Prior to determining whether the status was imprinted or LOI, the density ratio between the top and bottom bands, determined using PCR-RFLP, and the height ratio of the C and T peaks, examined using sequence analysis, were measured and calculated (Table I). Each case was identified as either imprinted or LOI, according to the criteria described above (Table I). LOI was detected in the adenocarcinoma and large cell carcinoma specimens, but was not detected in the squamous cell carcinoma samples. The percentage of LOI was 39% in total lung carcinomas (9 out of 23 cases) and 62% (8 out of 13 cases) in adenocarcinomas.

**Association between LOI and histological grade.** Since a previous study concerning IGF2 LOI in lung adenocarcinoma refers to histological type (7), the association between the histological grade of the adenocarcinoma sample and LOI was also analyzed in the present study. Four cases of well-differentiated adenocarcinoma were found to be LOI and one case of poorly differentiated adenocarcinoma was imprinted (Table I). These results do not support the previous suggestion that IGF2 LOI occurs more frequently in poorly differentiated adenocarcinomas (7). Statistical analysis revealed no significant association between the degree of differentiation and LOI.

**Discussion**

In the present study, the status of genomic imprinting of IGF2 in lung cancer was evaluated. IGF2 LOI was found to
Table I. IGF2 status of laser-capture-microdissected carcinoma cells, defined as imprinted or LOI in informative cases, evaluated by PCR-RFLP in combination with direct sequencing.

| Case number | PCR (%T) | Sequence (%T) | Status   | Histology     | Differentiation of adenocarcinoma |
|-------------|----------|---------------|----------|---------------|----------------------------------|
| 1           | 0        | 12            | Imprinted | SCC           |                                  |
| 2           | 72       | 44            | LOI      | Adenocarcinoma| Well                             |
| 8           | 54       | 25            | LOI      | Adenocarcinoma| Well                             |
| 10          | 77       | 33            | LOI      | Adenocarcinoma| Well                             |
| 11          | 0        | 17            | Imprinted| SCC           |                                  |
| 12          | 0        | 18            | Imprinted| SCC           |                                  |
| 13          | 62       | 69            | LOI      | Adenocarcinoma| Moderate                         |
| 14          | 73       | 41            | LOI      | Large cell carcinoma |                                  |
| 15          | 58       | 46            | LOI      | Adenocarcinoma| Moderate                         |
| 16          | 0        | 16            | Imprinted| Adenocarcinoma| Moderate                         |
| 17          | 0        | 16            | Imprinted| SCC           |                                  |
| 18          | 0        | 11            | Imprinted| Adenocarcinoma| Moderate                         |
| 19          | 9        | 17            | Imprinted| Adenocarcinoma| Well                             |
| 20          | 7        | 16            | Imprinted| Adenocarcinoma| Moderate                         |
| 21          | 5        | 16            | Imprinted| SCC           |                                  |
| 24          | 0        | 13            | Imprinted| SCC           |                                  |
| 25          | 0        | 3             | Imprinted| Adenocarcinoma| Poor                             |
| 26          | 83       | 62            | LOI      | Adenocarcinoma| Poor                             |
| 28          | 100      | 85            | Imprinted| SCC           |                                  |
| 29          | 60       | 35            | LOI      | Adenocarcinoma| Moderate                         |
| 30          | 0        | 22            | Imprinted| Adenocarcinoma| Moderate                         |
| 31          | 100      | 86            | Imprinted| SCC           |                                  |
| 32          | 0        | 9             | Imprinted| SCC           |                                  |

The density ratios of the top and bottom bands (bottom/top+bottom) in PCR-RFLP and the height ratios of T and C peaks (T/T+C) in sequencing are listed. When only one band was identified with PCR-RFLP (ratio 10-90%) or the sequence pattern revealed a single peak (ratio 20-80%), the status was considered to be LOI; otherwise, the status was considered to be imprinted. IGF2, insulin-like growth factor 2; LOI, loss of imprinting; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; SCC, squamous cell carcinoma.

Figure 1. Polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis using IGF2 gene ApaI polymorphism and sequencing for laser-capture-microdissected samples of lung carcinomas. The PCR products were digested with ApaI and run on 2% agarose gels. In the electropherogram, green indicates adenine; black, guanine; blue, cytosine and red, thymine.
increase IGF2 signaling by increasing the proliferation of expression-related genes (17). We hypothesize that IGF2 LOI leads to an increased risk of malignant transformation.

In this study, IGF2 LOI was detected in half the cases of adenocarcinoma, but not in any of cases of lung squamous cell carcinoma, which suggests an association between IGF2 LOI and the histological type of the tumor. A number of adenocarcinoma-specific gene alterations are known, including EGFR, KRAS and BRAF, and these affect the gene products of the MAP kinase and PI3/AKT signaling pathways (18,19). IGF2 binds to IR or IGF1R, thereby activating the same pathways (12). In this context, the effect of IGF2 overexpression appears comparable with the effect of other gene alterations specific to adenocarcinoma.

The incidence of IGF2 LOI in adenocarcinoma (62%) appears high as compared with other adenocarcinoma-specific gene alterations. However, the incidence of gene alteration may reflect population differences. A previous study indicated that the most frequent gene alteration in lung adenocarcinomas in Japanese patients is the EGFR mutation, with an incidence of 38%, while the most frequent gene alteration in Caucasians is KRAS mutation, with an incidence of 30% (19). The high incidence of the IGF2 mutation in the present study, as compared with the incidences of other genes detected in previous studies, suggests an important role for the IGF2 mutation in lung carcinogenesis.

In the present study, IGF2 LOI was detected in approximately half of adenocarcinomas but not in any of the squamous cell carcinomas examined; thus, IGF2 LOI may be a marker of lung adenocarcinoma. Distinguishing squamous cell carcinoma from adenocarcinoma of the lung is important, since the therapeutic methods employed are different and molecular analysis is guided by the histology. Molecular-targeted therapeutic drugs for activated EGFR have resulted in improvements in response rates and progression-free survival times in lung adenocarcinoma (20).

In conclusion, in the present study, IGF2 LOI was observed to occur at a high frequency in lung adenocarcinoma, but was not observed in squamous cell carcinoma. This result suggests that distinct carcinogenic pathways may exist for lung adenocarcinoma and squamous cell carcinoma, depending on the IGF2 genomic imprinting status. Therefore, IGF2 may have potential value as a diagnostic marker and therapeutic target.

Acknowledgements

This study was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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