Jpn. J. Cancer Res. **90**, 1244–1247, November 1999

Clonal Analysis of Adenosquamous Carcinoma of the Lung

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Adenosquamous carcinoma of the lung is a subset of pulmonary carcinomas, and comprises less than 4% of lung carcinomas. Its histogenesis remains unclear. The clonality of adenosquamous carcinoma from four female patients was analyzed to determine whether the clonality between the squamous cell and adenocarcinomatous components coincides. Each lesion was precisely microdissected from methanol-fixed sections. Adjacent normal lung tissue was collected as a normal control. DNA was extracted for clonal analysis based on an X-chromosome-linked polymorphic marker, the human androgen receptor gene (*HUMARA*). *HUMARA* was found to be amplified with or without previous digestion by the methylation-sensitive restriction endonuclease *Hpa*II. All four cases were informative. Squamous cell and adenocarcinomatous components showed identical monoclonal patterns in all four patients. In one case, only the squamous cell carcinomatous component showed loss of heterozygosity of the *HUMARA* locus. The results suggest that the squamous cell and adenocarcinomatous components originate from the same cell.

Key words: Adenosquamous carcinoma — Lung — Clonality — X-chromosome inactivation — Human androgen receptor gene

Adenosquamous carcinoma of the lung is a subset of pulmonary carcinomas, and comprises less than 4% of lung carcinomas.[1, 2] Some clinicopathological studies have suggested that adenosquamous carcinoma of the lung is more aggressive than adenocarcinoma or squamous cell carcinoma.[2–4] However, adenosquamous carcinoma has not been investigated genetically to clarify its histogenesis. On the basis of histology, there are many possibilities, including adenocarcinoma with squamous metaplasia, collision tumor, and bipotential undifferentiated cell origin.[3]

Monoclonality has been regarded as a fundamental characteristic of neoplasia.[5, 6] One X chromosome is randomly and permanently inactivated at an early stage of embryogenesis in the female. This leads to somatic mosaicism of normal females with respect to X-linked alleles, with approximately half of the somatic cells expressing the maternal allele and the other half expressing the paternal allele. Tumors arising from a single cell will therefore express one of the two phenotypes. Recently, a highly polymorphic trinucleotide CAG repeat in the X-linked human androgen receptor gene (*HUMARA*) has been used to distinguish between the two X chromosomes[7] and to detect monoclonality in various neoplastic diseases.[8–14] Clonal analysis is one of the most useful methods to determine whether two components have the same origin.[15]

The aim of this study was to determine whether the squamous cell and adenocarcinomatous components of adenosquamous carcinoma exhibit identical monoclonality.

MATERIALS AND METHODS

Patients Two hundred and one patients underwent resection of the lung because of a tumor at the National Cancer Center Hospital East from September 1997 to August 1998. Sixteen (8%) tumors were adenosquamous carcinomas (11 males and 5 females) (Table I). Tissue from four female cases was used in the analysis of the clonality.

Histologic criteria for adenosquamous carcinoma The histologic diagnosis of adenosquamous carcinoma was made based on the following criteria, as previously described.[3, 16] 1) The lesion shows unequivocal squamous differentiation in the form of keratin or intercellular bridges, and unequivocal glandular differentiation in the form of acini, tubules, or papillary structures. 2) Each component occupies at least 5% of the tumor area (Fig. 1).

Strategy for clonal analysis We conducted clonal analysis according to the method of Allen *et al.*[7] The strategy is based on random X chromosome inactivation by methylation. This random inactivation occurs early in embryogenesis, and remains conserved throughout cell division, even in tumors. The *HUMARA* on the X chromosome has a trinucleotide (CAG) repeat polymorphism. We used *Hpa*II, which cannot digest methylated DNA, to cut DNA before polymerase chain reaction (PCR), so that only the methylated allele was amplified. If the methylation pattern is

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Table I. Patients Who Underwent Resection of the Lung because of a Tumor at the National Cancer Center Hospital East from September 1997 to August 1998

Pathologic classification	No. of patients	Percentage	Male/ female
Adenocarcinoma	104	52	55/49
Squamous cell carcinoma	37	18	31/6
Adenosquamous carcinoma	16	8	11/5
Large cell carcinoma	5	2	5/0
Small cell carcinoma	3	2	1/2
Tumors metastatic to the lung	30	15	18/12
Others	6	3	5/1

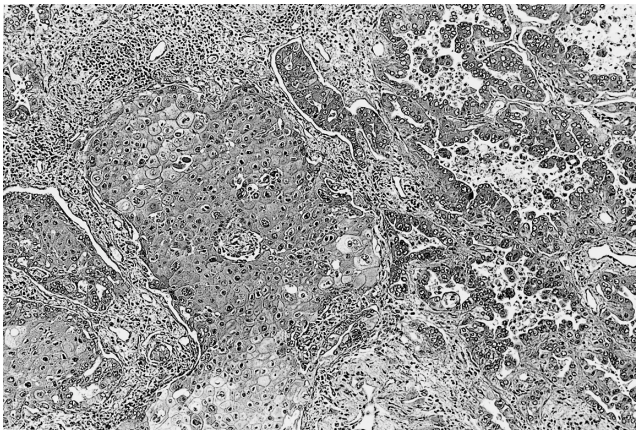

Fig. 1. Representative histopathological features of adenosquamous carcinoma. A well-defined nodule was detected in the peripheral lung. Cuboidal to low-columnar tumor cells are arranged in a papillary manner at the periphery and midzone. Polygonal tumor cells with intercellular bridges form nests in the central part of the tumor (H&E, original magnification, ×90).

uniform because of monoclonality, there is only one PCR product after *Hpa*II digestion, whereas if the methylation patterns differ (polyclonal), two PCR products are obtained for the trinucleotide repeat polymorphism. The *HUMARA* polymorphism results were compared to the normal sample used in the study. In monoclonal cases, if the longer or shorter allele is amplified after *Hpa*II digestion, we refer to it as the "l" or "s" pattern of monoclonality, respectively.

Microdissection of materials from stained slides and DNA extraction The resected lungs were fixed with 100% methanol and embedded in paraffin. The paraffin blocks containing adenosquamous carcinoma that was found in routine pathological studies were cut into 5-μm sections and stained with hematoxylin and eosin. A precise microdissection technique was performed according to a method previously described.[17] Approximately 1,000 cells from the squamous cell or adenocarcinomatous components were microdissected separately under a micromanipulator (Olympus, Tokyo). Adjacent normal lung tissue was scraped with a 27-gauge needle to provide a normal control. DNA was extracted with a WB DNA extractor kit (Wako Pure Chemicals, Osaka).

PCR DNA was digested with *Rsa*I and with or without *Hpa*II, and the *HUMARA* was then amplified by PCR as previously described.[18] Amplification of the *HUMARA* in exon 1 was performed using primers AR1 and AR2, essentially as described by Mutter and Boynton[19] with slight modifications. Dimethylsulfoxide (DMSO; 6% w/v) and dGTP instead of 7-deaza-2′-dGTP were added. AR2 was labeled at the 3′ end with fluorescein. The *HUMARA* PCR products were analyzed with an automatic sequencer (ALFred; Pharmacia, Uppsala, Sweden) and quantified with a Fragment Manager software package (Pharmacia).

RESULTS

The characteristics of the cases are summarized in Table II. Lobectomy and mediastinal lymph node resection were performed in all cases. The proportion of the squamous cell carcinoma component in a tumor varied from 10 to 50%. Nodal involvement was detected in case 3, in which only an adenocarcinomatous component was identified. A papillary subtype of adenocarcinomatous component was found in cases 1, 2, and 4, whereas an acinar subtype was found in case 3.

All four cases were informative, with two PCR products of *HUMARA* (s and l) in the normal control. In case 2, loss of heterozygosity (LOH) in the *HUMARA* locus was found only in the squamous cell carcinomatous component, whereas the adenocarcinomatous component exhibited an l pattern of monoclonality (Fig. 2). The *HUMARA* was not amplified in the squamous cell carcinomatous component in case 2 after *Hpa*II digestion. This meant that retained allele was not methylated and was digested by *Hpa*II. Therefore, the squamous cell carcinomatous component in case 2 showed an l pattern of monoclonality, identical to the adenocarcinomatous component. Accordingly, all four cases showed an identical monoclonal pattern between the squamous cell and adenocarcinomatous components. Normal lung showed a polyclonal pattern.

DISCUSSION

Adenosquamous carcinoma of the lung is an infrequent tumor, and no genetic analyses have been reported to our knowledge. An immunohistochemical analysis showed different expression characteristics for proteins such as keratin, carcinoembryonic antigen, epithelial membrane antigen, secretory component, and lactoferrin, between squamous cell and adenocarcinomatous components.[20]

Table II. Clinical Features and Clonal Results

Case	Age (years)	TNM	Percentage of each component in the tumor[a)]	Histologic type	Subtype	*HUMARA*
1	63	T1N0M0	10	sq		Monoclonal (s)
			90	ad	Papillary	Monoclonal (s)
				NL		Polyclonal
2	83	T2N0M0	30	sq		LOH
			70	ad	Papillary	Monoclonal (l)
				NL		Polyclonal
3	58	T4N2M0	20	sq		Monoclonal (l)
			80	ad	Acinar	Monoclonal (l)
				NL		Polyclonal
4	71	T3N0M0	50	sq		Monoclonal (s)
			50	ad	Papillary	Monoclonal (s)
				NL		Polyclonal

a) The percentage of each component in a lesion was determined microscopically. sq, squamous cell carcinomatous component; ad, adenocarcinomatous component; NL, normal lung; s, shorter-allele-inactivated pattern; l, longer-allele-inactivated pattern.

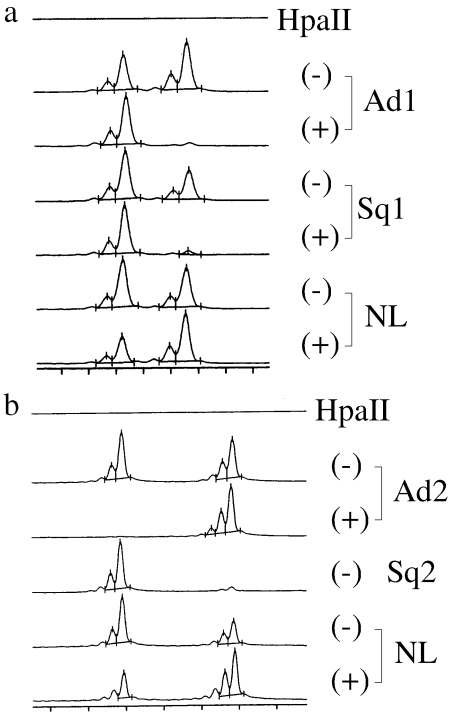

Fig. 2. Clonal analysis by amplification of the human androgen receptor gene with or without *Hpa*II digestion in case 1 (a) and case 2 (b). Both the squamous cell and adenocarcinomatous components showed s pattern of monoclonality in case 1. On the other hand, the squamous cell carcinomatous component showed an LOH at the *HUMARA* locus, whereas the adenocarcinomatous component exhibited l pattern of monoclonality in case 2. Ad, adenocarcinomatous component; Sq, squamous cell carcinomatous component; NL, normal lung.

We demonstrated that squamous cell and adenocarcinomatous components showed an identical monoclonal pattern in all four cases. These findings support the hypothesis that each component had the same origin. However, an identical monoclonal pattern would occur 50% of the time by chance alone in one case, 25% in two cases, 12.5% in three, and 6.25% in four. Therefore, clonal analysis of many cases is needed to determine the histogenesis of adenosquamous carcinoma of the lung.

Only the squamous cell carcinomatous component showed LOH at the *HUMARA* locus in case 2. This notable finding suggests that tumorigenesis of the squamous cell carcinomatous component was a later event than that of the adenocarcinomatous component, if one tumor type differentiated into another component. Histologically, adenosquamous carcinoma includes adenocarcinoma with squamous metaplasia, collision tumor, and bipotential undifferentiated cell origin. Our finding genetically supports the idea that a part of the adenocarcinoma differentiated into squamous cell carcinoma in some adenosquamous carcinomas. Adenosquamous carcinoma is known to show not only histological, but also genetic intratumor heterogeneity. LOH at the *HUMARA* locus has been rarely described, except in glioma.[21)] The significance of LOH at the *HUMARA* locus in adenosquamous carcinoma has yet to be determined. Allelic loss could be a random event related to genetic instability with partial or complete deletion of the X-chromosome or could target a tumor suppressor gene adjacent to the *HUMARA* locus.[21)] Further clonal analyses may resolve this problem.

The histologic classification of the World Health Organization (WHO) states that adenosquamous carcinoma is

composed of both squamous cell and adenocarcinomatous components.[16] While the proportion of each component required for the diagnosis is not defined, a minimum amount of 5% for one component is reasonable.[3] We also used 5% as a minimum criterion. The incidence of adenosquamous carcinoma was 8% in our series, which is slightly higher than in previous reports.[1–4, 20] The discrepancy is probably not significant because the reported incidence of adenosquamous carcinoma depends on the diagnostic criteria.

In conclusion, squamous cell and adenocarcinomatous components showed identical monoclonal patterns. In one case, only the squamous cell carcinoma component showed LOH of the *HUMARA* locus. The results suggest that the squamous cell and adenocarcinomatous components are derived from the same cell.

ACKNOWLEDGMENTS

We wish to thank Professor Michio Yamakido, The Second Department of Internal Medicine, Hiroshima University School of Medicine, Hiroshima, Japan, for his encouragement during this study. This work was supported in part by a Grant from the Ministry of Health and Welfare for the 2nd Term Comprehensive Strategy for Cancer Control and a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan.

(Received July 6, 1999/Revised August 6, 1999/Accepted August 10, 1999)

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