BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **221,** 122–128 (1996)
ARTICLE NOS. 0556

The Clonal Progression in the Neoplastic Process of Nasopharyngeal Carcinoma

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Received February 26, 1996

The clonality of a total of 70 human nasopharyngeal carcinomas (NPC) was analyzed using the structure of the terminal fragment of episomal Epstein-Barr virus (EBV). Thirty female samples heterozygous for the BstXI polymorphism of the phosphoglycerokinase (PGK) gene were analyzed using polymerase chain reaction (PCR) amplification of X-chromosome linked PGK gene for restriction fragment length polymorphism (RFLP). All NPC samples analyzed were shown to be monoclonal, with two exceptions that were polyclonal. Clonal determination was also performed for non-cancerous cell populations: normal, and simple hyperplastic, grade I (mild) and grade II–III (severe) atypical hyperplastic epithelia. It was found that the normal and simple hyperplastic and 3 grade I (mild) atypical hyperplastic epithelia were polyclonal, whereas the grade II–III (severe) atypical hyperplastic samples were monoclonal. The analysis of the clonality of various stages in the neoplastic process suggested that NPC might originate from several cells, after clonal selection; finally a large majority of NPC has been demonstrated to be monoclonal, also indicating that the alteration of clonal nature might have occurred at a very early stage.

Most human cancers have been found to be monoclonal (1–6), as predicted by the somatic mutation theory of carcinogenesis (7). A few types of tumors (8–10), however, were identified to be polyclonal, consistent with the argument of cell autocrine growth theory (11–12). Moreover, cancers induced by chemical carcinogenesis in animal experiments also appeared to be polyclonal (13,14). The controversy of these reported experimental results on human tumor clonality is believed to be due to the complexity of malignant transformation (15,16) and the fact that only the clonality of middle and advanced tumors was analyzed in the previous studies. It is very likely that further mutation and selective clonal expansion within these tumors have already occurred, and hence the clonal analysis of these samples would not convey any information on the clonal progression in the earlier stages of neoplastic process. In addition, the contamination of tumor tissues by the adjacent polyclonal non-tumorous cells, which sometimes is unavoidable, make the interpretation of clonal analysis results of tumor cells more difficult and sometimes even misleading.

The recently developed clonal analysis method was based on restriction fragment length polymorphism (RFLP) of X-chromosome linked PGK and on random gene inactivation by methylation (17). This approach greatly increased the scope of clonality studies to all females who have a suitable X-linked DNA polymorphism. With the introduction of polymerase chain reaction (PCR), Gilliland et al (18) modified this method and applied it to the clonal analysis of a small number of cells. Using the microdissection technique in conjunction with this PCR-based method, it is now possible to isolate and analyze the clonality of different cell populations of neoplastic process (19,20). Such studies will be provide an additional and helpful approach to understand the pathogenesis of the malignancies (21).

Nasopharyngeal carcinoma is a prevalent cancer in Southern China, but only a few reports have

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Abbreviations used: NPC, nasopharyngeal carcinoma; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; PGK, phospholycerokinase. G6PD, glucose-6-phosphate dehydrogenase.

0006-291X/96 $18.00

been published on its clonality. Fiallow et al (2) first analyzed the clonality of NPC using the X-chromosome linked glucose-6-phosphate dehydrogenase (G6PD) isoenzyme as a marker, but and the results were quite ambiguous. Raab-Traub and Flynn (3) determined NPC to be monoclonal using the structure of the termini of Epstein-Barr virus as a marker. However, the clonal origin of NPC is still not clear since the samples they tested were extracted from advanced biopsy tissues of NPC. In order to study the clonal development of NPC, we analyzed the clonality of different cell populations during the neoplastic process using the microdissection technique in conjunction with PCR-based X-linked RFLP analysis. The clonality of NPC were also determined using the fused terminus of the latent episomal EBV as a marker. Our results suggest that NPC might originate from several cells, after clonal selection, finally a large majority of NPC has been demonstrated to be monoclonal, also indicated the alteration of clonal nature might have occurred at a very early stage.

MATERIALS AND METHODS

Tissue source. Most of nasopharyngeal biopsy tissues were provided by the Out-Patient Department in Hunan Tumor Hospital. Some samples were obtained from the First Affiliated Hospital of Henan Medical University (10 females) during the period between September 1991 and March 1994. The histological type of the samples was classified as normal, simple hyperplasitic, atypical hyperplasitic epithelia and cancerous foci according to the World Health Organization histological classification (19). The atypical hyperplasitic epithelium was described as the grade I (mild), II (moderate) and III (severe) on the basis of the degree of cellular atypia.

Microdissection and DNA extraction. Before DNA extraction, the female biopsy tissues were embedded in OCT compound and snap-frozen in liquid nitrogen for frozen dissection and microdissection. For each female biopsy tissue, one 5μm-section and five 10μm-sections were serially cut on glass slides with a cryostat. The 5μm-section was stained with HE for histological diagnosis and the five 10μm-sections with Giemsa to identify histological structures for microdisection. It was observed in this study that Giemsa staining had much less effect on the efficiency of PCR as compared to HE staining. The miocodissection was performed using a disposable modified 30-gauge needle under a 25-times inverted light microscope. The DNA samples were treated with 40μl of a mixed solution of 50mMKCl, 10mMTris/HCl, 2.5mMMgCl_2, 0.45%P40, 45%Tween-20, and Proteinase K 200μg/ml, followed by phenol/chloroform extraction for PCR. The male biopsy tissues and the remainder of female biopsy tissues were subjected to Proteinase K digestion and phenol/chloroform extraction. The extracted DNA was suspended in TE(pH8.0) for Southern hybridization.

Selection of the patients and clonal analysis by PCR. The clonal analysis of different cell populations was performed using the method as described by Gilliland et al and the strategy of clonal analysis is shown in Fig. 1. A 20μl of DNA solution extracted from the cryostat sections was amplified in 50μl PCR reaction solution of 50mMKCl, 10mMTris/HCl(pH8.3), 1.5mMMgCl_2, dNTP(200M each), 20pmol primer1A, 20pmol primer1B and 2.5 unit of Taq DNA polymerase (Sigma) PCR was performed for 30 cycles. Each cycle consisted of 50 sec at 94°C, 50 sec at 58°C and 1 min at 72°. The 5μl of this PCR mixture was transferred into another reaction tube and mixed with 20 pmol of each Primer2A and Primer2B. The amplification was then continued for another 30 cycles as described above. After amplification, one-tenth of the solution was digested with 2 unit BstXI for 6hrs at 50°C. The 2% agarose gel electrophoresis was used to analyze the PCR amplified products. The sample heterozygous for BstXI polymorphism of the PGK were selected for further analysis, which were incubated in the presence of 5 units of HpaII (Sigma) at 37°C for 12hrs and then at 100°C for 3 min. The first and the nested second PCR, subsequent digestion with BstXI and agarose gel electrophoresis of the amplified products were performed following the same procedure as described above.

Clonal analysis by Southern blotting. Purified DNA was digested with BamHI (5 unit per/DNA) under the conditions recommended by the manufacturer. Digested DNA was loaded (10μg per land) into 0.8% agarose gels. Electrophoresis of the DNA was conducted at an electric field of 1 $\sim$ 1.5 V/cm for 16 hours. The DNA then was transferred to nylon membranes and hybridized using the [^{32}P] dCTP random prime labeled LMP probe of XhoII.9kb (2). This probe represented the unique DNA adjacent to the right terminal repeat sequence of EBV. The hybridized blot was exposed to X-ray films with an intensifying screen.

RESULTS

Clonal analysis of nasopharyngeal biopsy tissues using the clonal marker of the structure of terminal repeat of EBV. A total 70 of NPC samples were analyzed for their clonality using the structure of termui repeat of EBV. The representative results are shown in Fig. 1. The control sample, B95-8 Raji cell line, was simultaneously analyzed. It was found that the polyclonal B95-8 and the DNA contained four large fragments ranging in size from 8 to 10kb, and five small

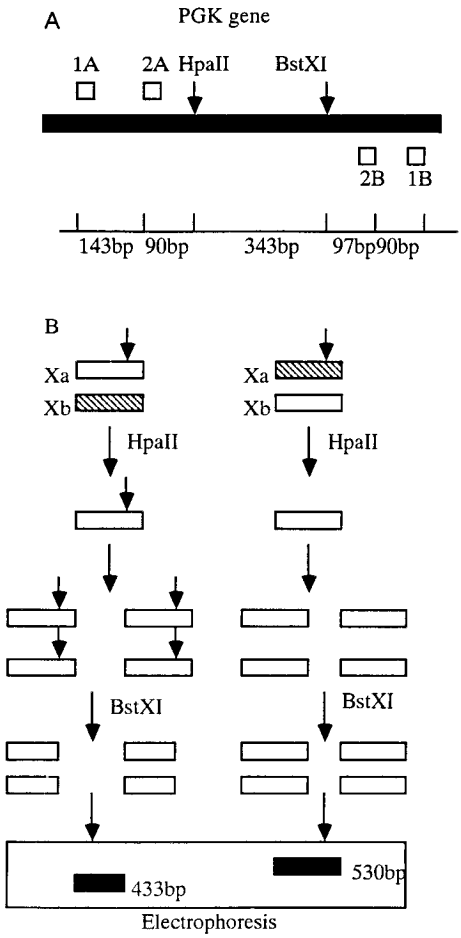

FIG. 1. (A) Restriction map of PGK gene containing differentially methylated HapII site and polymorphic BstXI site. DNA sequences of primers used for the amplification by PCR were: 1A: 5′-CTGTTCCTGCCCGCGCGGTGTTCCG-CATTC-3′, 1B: 5′-ACGCCTGTTACGTAAGCTTGCAGGCCTCC-3′, 2A: 5′-AGCTGGACGTTAAAGGGAAGC-GGGTCGGTTA-3′, 2B: 5′-TACTCCTGAAGTTAAATCAACATCCTCTTC-3′ (B) Schematic drawing of the strategy for the PCR-based method of clonal analysis. Xa represents an X chromosome that contains a PGK gene with a BstXI restriction site(arrow); Xb represents an X chromosome that contains a PGK gene without a BstXI restriction site. Somatic cells of females heterozygous for the BstXI polymorphism of the PGK gene are composed of two types of cells i.e., those with inactive X and those with inactive Xb due to random inactivation of one of two X chromosomes by methylation. Active X chromosomes (hatched bar) are cleaved after methylation-sensitive HpaII digestion. Methylated, inactive PGK allele is preserved after the HpaII digestion and amplified by PCR. The PCR-amplified product is then digested with BstXI and analyzed by agarose gel electrophoresis.

fragments ranging in size from 4 to 6kb. The large fragments were believed to represent the fused termini and the small ones the linear replication of EBV. In contrast, the monoclonal Raji cell line contained only one large 20kb terminal fragment. Of the total 70 NPC biopsy tissues (10 male and 60 female) analyzed, 10 male and 58 female samples were found to have a single large terminal fragment. The fragment size varied from 8 to 12kb due to the variation of reiteration of the TR sequences of EBV from sample to sample. The results suggested that these NPC samples contained only one form of episome of EBV and were monoclonal.

The other two female samples (cases 41 and 30), however, contained more than one large fragment, indicating the presence of two forms of fused termini. These two samples were believed to be polyclonal.

One male sample (case 28) had an additional three small fragments (<5kb) representing linear replicating DNA of EBV. No EBV genome was detected in normal and simple hyperplsia nasopharyngeal tissues.

Clonal analysis of the different stages of neoplastic process using X-chromosome RFLP. The DNA samples extracted from cryostat sections were first tested for their heterozygosity for the BstXI polymorphism of the PGK gene. Of total 90 female patients analyzed, 30 were found to be heterozygious, that is, without HapII digestion, their PCR-amplified and BstXI-digested products gave rise to 2 DNA bands, 530 bp (allele 1) and 433 bp (allele2). The DNA samples extracted from different cell populations, which were separated from each other by microdissecting these 30 cryostat section, were then digested with HapII and analyzed for their clonality using PCR based X-chromosome RFLP. The results are summarized in Table 1 and some representatives are shown in Fig. 2.

It was observed that, when digested with HpaII prior to PCR, the normal, simple hyperplastic and grade I atypical hyperplastic epithelia samples gave rise to 2 bands, allele 1 and allele 2, and were polyclonal, regardless of their locations either adjacent to the cancerous foci or not. The grade II $\sim$ III atypical hyperplastic epithelia adjacent to the cancerous foci only gave rise to one band, allele 1 or allele 2, and were monoclonal. The cancerous foci, with one exception, were also showed to be monoclonal. One cancerous foci sample (case 30), however, showed polyclonality, from which both bands, allele 1 and allele 2, were observed. For the 20 female NPC samples analyzed, the clonality determined using PCR based X-chromosome RFLP were the same as that determined by the structure of the termini of EBV.

It was noticed that the grade I atypical hyperplastic epithelia in sample 19 showed a very weak allele 2 (433bp) band, as compared to that of its adjacent normal epithelia. This implied that a predominant clone was developed in this stage of cell populations.

TABLE 1
The Clonal Analysis Results Using PCR-Based X-chromosome RFLP and the Structure of the Termini of EBV

		Clonal results analysis	
Patient ID	Histological type	X-chromosome RFLP	The termini of EBV
1–3	Normal epithelism	Polyclonal	—
4–7	Simple hyperplastic epithelism	Polyclonal	—
7–9	Grade I atypical hyperplastic epithelism	Polyclonal	—
10	Normal epithelism	Polyclonal	
	Grade I atypical hyperplastic epithelism	Polyclonal	
	Grade II$\sim$III atypical hyperplastic epithelism	Monoclonal	
	Cancerous foci	Monoclonal	Monoclonal
11–13	Normal epithelism	Polyclonal	
	Cancerous foci	Monoclonal	Monoclonal
14–17	Simple hyperplastic epithelism	Polyclonal	
	Cancerous foci	Monoclonal	Monoclonal
18,19	Normal epithelism	Polyclonal	
	Grade I atypical hyperplastic epithelism	Polyclonal	
	Cancerous foci	Monoclonal	Monoclonal
20	Grade II$\sim$III atypical hyperplastic epithelism	Monoclonal	
	Cancerous foci	Monoclonal	Monoclonal
21	Normal epithelism	Polyclonal	
	Grade II$\sim$III atypical hyperplastic	Monoclonal	
	Epithelism	Monoclonal	Monoclonal
	Cancerous foci		
22–29	Cancerous foci	Monoclonal	Monoclonal
30	Normal epithelism	Polyclonal	
	Cancerous foci	Polyclonal	Biclonal

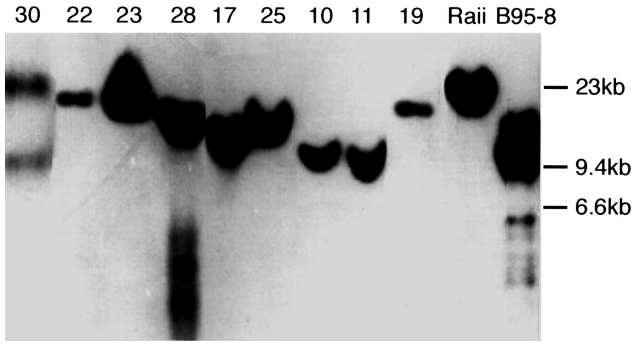

FIG. 2. Analysis of EBV Termini in NPCs. DNAs obtained from the Raji and B95-8 cell lines and from biopsy tissues of NPCs were digested with BamHI, subjected to electrophoresis through a 0.8% agarose gel, transferred to nitrocellulose, and hybridized to the p32-XhoI1.9kb fragment representing unique DNA adjacent to the right terminal repeats.

DISCUSSION

The clonality of a total of 70 human NPC samples was analyzed using the structure of terminus repeat of EBV, 68 NPC were found to be monoclonal and two were polyclonal. Of total 70 NPC samples, 20 female NPC samples were heterozygous for the BstXI polymorphism and were also analyzed their clonality using the PCR based X-chromosome RFLP. Both methods yielded identical results, suggesting that the two methods were equalivantly reliable in clonal determination for NPC. On the other hand, the detection of homogeneous episomal EBV DNA in NPC suggested that the virus played a primary role in the pathogenesis of NPC.

There are increasing evidences that the pathogenesis of cancer proceeds in sequential steps from

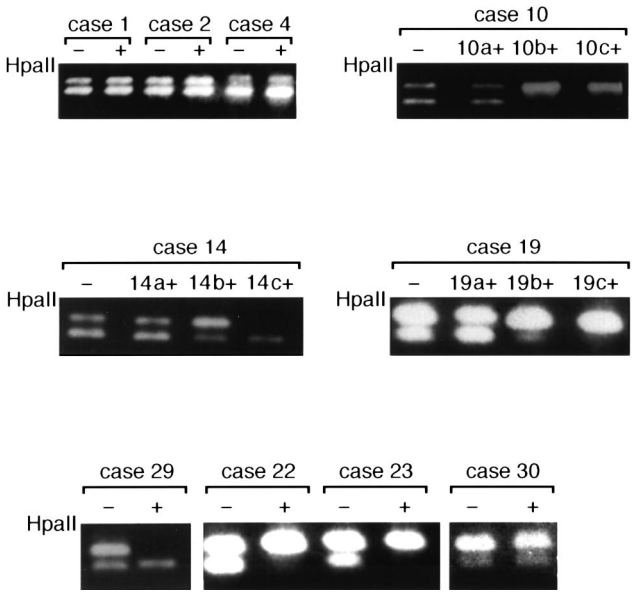

FIG. 3. Clonal analysis of the different stages of neoplastic process of NPC. DNA samples were prepared from cryostat sections of case1, case2 (normal epithelia); case4 (simple hyperplastic epithelia); case 22, 23, 29, 30 (cancerous foci); case 10, 14, 19 contained different cell populations adjacent to each other, respectively, 10a (normal), 10b (grade I atypical hyperplastic), 10c (cancerous foci); 14a (normal), 14b (simple hyperplastic), 14c (cancerous foci); 19a(normal), 19b (grade I atypical hyperplastic), 19c (cancerous foci). DNA was digested (+) or not digested (−) with HpaII prior to PCR. Clonal analysis of these samples was done as described in Materials and Methods. Results of agarose gel electrophoresis are shown.

TABLE 2
The Clonality of Various Stages in Neoplastic Process of NPC

Histopathological types	Clonality
Normal epithelia	Polyclonal
Simple hyperplastic epithelia	Polyclonal
I atypical hyperplastic epithelia	Polyclonal
II–III atypical hyperplastic epithelia	Monoclonal
Cancerous foci	Monoclonal

normal cells to premalignant foci, to located tumors and to invasive tumors (24). In order to trace the clonal progression of NPC, the clonality of various stages of cancerous process were analyzed using the PCR based X-chromosome RFLP. In some cases (10, 18, 19, 20, 21) analyzed, the different cell populations from normal epithelia to cancerous foci were adjacent to each other in one cryostat section. This provided a convincing model to study the clonal progression of NPC. The results reported here suggest that the revelation of the clonal progression of nasopharyngeal epithelia, as schematically illustrated in Table 2, can help to understand the pathogenesis of NPC. The poly-to-mono clonality transition occurred in a severe atypical hyperplastic lesion, and the transition might even be initiated in a mild or moderate atypical hyperplastic lesion. Sheu et al. reported recently that over expression of p53 was associated with the transition from dysplastic lesions to carcinoma and occurred at an early stage in the development of NPC (25). It was suggested that the alteration of clonal nature in an atypical hyperplastic stage, which presumably resulted from p53, bcl-2 (26) and/or other genetic changes, provided a cell with the selective advantage of allowing its descendants to become the predominant clone, and led to the evolution of clonal nature from polyclonality to monoclonality. But under few circumstance, malignant NPC still appeared two or more predominant clones. The analysis of the clonality of different stages in neoplastic process suggested that NPC might originate from several cells, after clonal selection, finally a large majority of NPC has been demonstrated to be monoclonal and also indicated the alteration of clonal nature happened at a very early stage, probably at atypical hyperplastic stage. In order to further understand the molecular basis for the clonal progression of NPCclonal progression and the pathogenesis of NPC, the genetic changes in various stages should be analyzed.

ACKNOWLEDGMENTS

The authors thank Dr. Ya Zhao of Hunan Medical University, and Pro Gui-Tin, Liu of Henan Medical University and Dr. P. Epstein of University of Connecticut Health Center for helpful advice. We also thank the Nasopharyngeal Division and Pathological Department in Hunan Tumor Hospital and the First Affiliated Hospital for providing nasopharyngeal biopsy tissues and helping to make pathological diagnoses.

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