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Genotypic Differentiation of Intrahepatically Transplanted Hyperplastic Nodule Cells of Analbuminemic and Normal Rat Origin by Polymerase Chain Reaction

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DNA fragments encompassing the region of the seven-base-pair deletion in the albumin gene, which is a characteristic abnormality of Nagase's analbuminemic rat (NAR), were amplified by polymerase chain reaction, and we could differentiate the genotypes of normal rat, homozygous NAR and heterozygous NAR electrophoretically. This genotyping method was applied to the differentiation of hyperplastic nodules on immunostained tissue sections in the intrahepatic transplantation model. When the hyperplastic nodule cells of normal rat were transplanted to the livers of homozygous NAR, the donor cells were effectively differentiated from the host cells by the genotype.

Key words: Nagase's analbuminemic rat — Hyperplastic hepatic nodule — Intrahepatic transplantation — Polymerase chain reaction — Genotypic marker

Nagase's analbuminemic rat (NAR) is a mutant breed which is genetically defective in the ability to produce albumin.[1] We previously established an experimental model of intrahepatic transplantation of albumin-negative hyperplastic nodule cells of homozygous NAR into the albumin-positive liver of heterozygous NAR.[2] This model enabled us to identify the fate of the transplanted hyperplastic nodules by albumin-immunohistochemistry. However, it is known that, upon treatment with some chemical carcinogens and during the process of aging, homozygous NAR hepatocytes become albumin-positive,[3] and the heterozygous NAR hepatocytes become albumin-negative.[4] Furthermore, it has been reported that hepatocellular carcinomas induced by chemicals in normal rats occasionally show reduced capacity to produce albumin.[5,6] So phenotypic expression of albumin is not an absolutely reliable marker in the above model.

Esumi *et al.*[7] reported that, in NAR, there was a seven-base-pair (bp) deletion at the start of the 9th intron which presumably blocked albumin mRNA splicing and resulted in defective albumin expression. This genetic abnormality is considered to be a reliable marker to distinguish the transplanted nodule cells. We present here a useful method to identify the 7-bp deletion by use of the polymerase chain reaction (PCR),[8] which enables us to distinguish the genotypes of normal rat, homozygous NAR and heterozygous NAR.

First, genomic DNAs were extracted from livers of Fischer 344 (F344), homozygous NAR and heterozygous NAR (F344×NAR F1), and used as templates for PCR. The primers for PCR were synthesized by the solid-phase method with the use of a DNA synthesizer (Waters). The primer A corresponds to 21 nucleotides within the 9th exon (exon H), and the primer B corresponds to 20 nucleotides within the 9th intron of the rat albumin gene (Fig. 1). The segment between these primers encompasses the 7-bp deletion found in NAR's albumin gene. When primers A and B were used for PCR, a 67-bp fragment was amplified from normal rat, a 60-bp fragment from homozygous NAR, and both 67-bp and 60-bp fragments from heterozygous NAR. These fragments were easily distinguished by the electrophoretic pattern, and 3 different genotypes were easily differentiated (Fig. 2). Direct sequencing of the PCR products with *Taq* polymerase[9] revealed that the DNA fragments amplified from the normal genome and those of NAR actually corresponded to the sequences of albumin gene of normal rat and NAR reported by Esumi *et al.*[7] (Fig. 3). Thus, this method was proved to be effective to differentiate the genotypes of normal rat, homozygous NAR and heterozygous NAR.

Then, we attempted to apply these methods to the differentiation of genotypes on tissue sections in the intrahepatic transplantation model. We used 2 different combinations of animals for the model. As analbuminemia of NAR is an autosomal recessive trait, the livers of heterozygous NAR (F344×NAR F1) are albumin-positive.[1,10] When F2 generation was produced by mating the heterozygous F1, about one-fourth of them were homozygous for analbuminemia. We performed intrahepatic transplantation of the hyperplastic nodule cells from NAR to heterozygous F1 (model 1) and from

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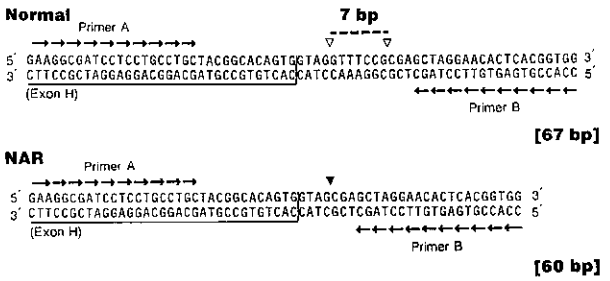

Fig. 1. DNA fragments amplified by PCR using primers A and B. A 67-bp DNA fragment spanning exon H and the ninth intron of rat albumin gene is amplified in normal rat. However, in NAR, a DNA fragment of smaller size (60-bp) is amplified due to a 7-bp deletion.

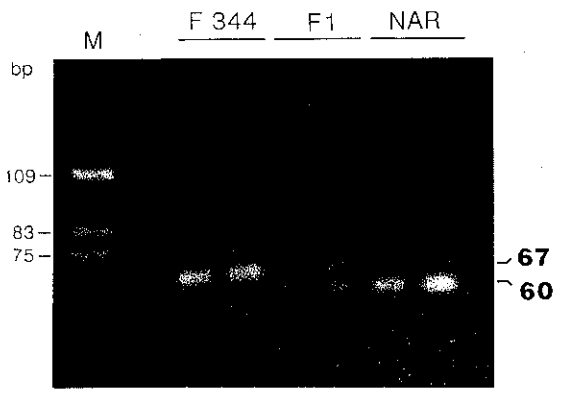

Fig. 2. Ethidium bromide staining of amplified products from genomic DNAs of normal rat (F344), F344×NAR F1 (heterozygous NAR) and NAR (homozygous NAR). A 67-bp fragment, a 60-bp fragment and both fragments were amplified from normal rat, homozygous NAR and heterozygous NAR, respectively. Staining was carried out after electrophoresis in a 4% NuSieve agarose mini-gel. M: molecular marker (*Hin*fI-digested pSV2cat).

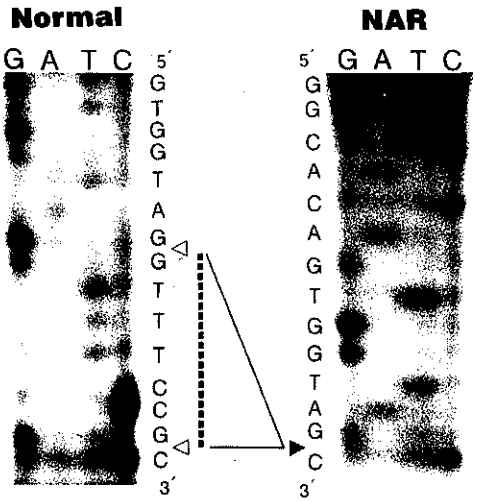

Fig. 3. Direct sequencing of the PCR products. First, single-stranded DNAs were generated by an asymmetric PCR (primer A:primer B=50 pmol:1 pmol) from genomic DNAs of normal rat and homozygous NAR. Then, using ^{32}P-labeled primer B as a sequencing primer, direct sequencing was performed. A 7-bp deletion (5′GTTTCCG3′) in NAR was confirmed.

F344 to homozygous F2 (model 2). It was proved that 100% of transplantation in the former case and about 75% in the latter case were successful. Hyperplastic nodules were induced in donor rats by Solt and Farber's regimen.[11] At the 6th week after the start of the regimen, the liver cells were isolated by collagenase perfusion. Host rats were given dietary 2-acetylaminofluorene (2-AAF) (0.02%) for 2 weeks and subjected to a two-thirds hepatectomy at one week from the start of 2-AAF feeding. They were immediately infused with the liver cells of donor rats from the mesenteric vein. Host rats were killed under light ether anesthesia at 2 weeks after transplantation. The livers were fixed with periodate-lysine-paraformaldehyde fixative and embedded in paraffin. Serial thin sections were immunostained for albumin and glutathione *S*-transferase placental form (GST-P) by the biotin-streptoavidin method. In model 1, the majority of the GST-P-positive hyperplastic nodules were albumin-negative, while in model 2, the majority of the hyperplastic nodules were albumin-positive. However, there were some albumin-positive nodules in model 1 and albumin-negative ones in model 2 (Fig. 4).

Under stereoscopic observation, individual nodules were isolated by using a syringe needle from several immunostained serial sections. The collected tissue fragments were heated at 94°C for 7 min and used as PCR templates without extraction of DNAs. The hepatic tissue surrounding the nodules was also collected and used as a control. In model 1, the 60-bp band of donor (NAR) origin was predominant as compared with the 67-bp band of normal rat origin in the albumin-negative nodules in the PCR using the primers A and B (Fig. 5a). In model 2, the band of donor (F344) origin was detected in addition to the band of NAR origin in the albumin-positive nodules, while only the band of host (NAR) origin was demonstrated in the surrounding host liver and albumin-negative nodules (Fig. 5b). We repeated the experiment using 16 nodules in model 1 (10 albumin-negative nodules and 6 albumin-positive nodules), and 32 nodules (24 albumin-positive nodules and 8 albumin-negative nodules) in model 2 and confirmed that the results were reproducible.

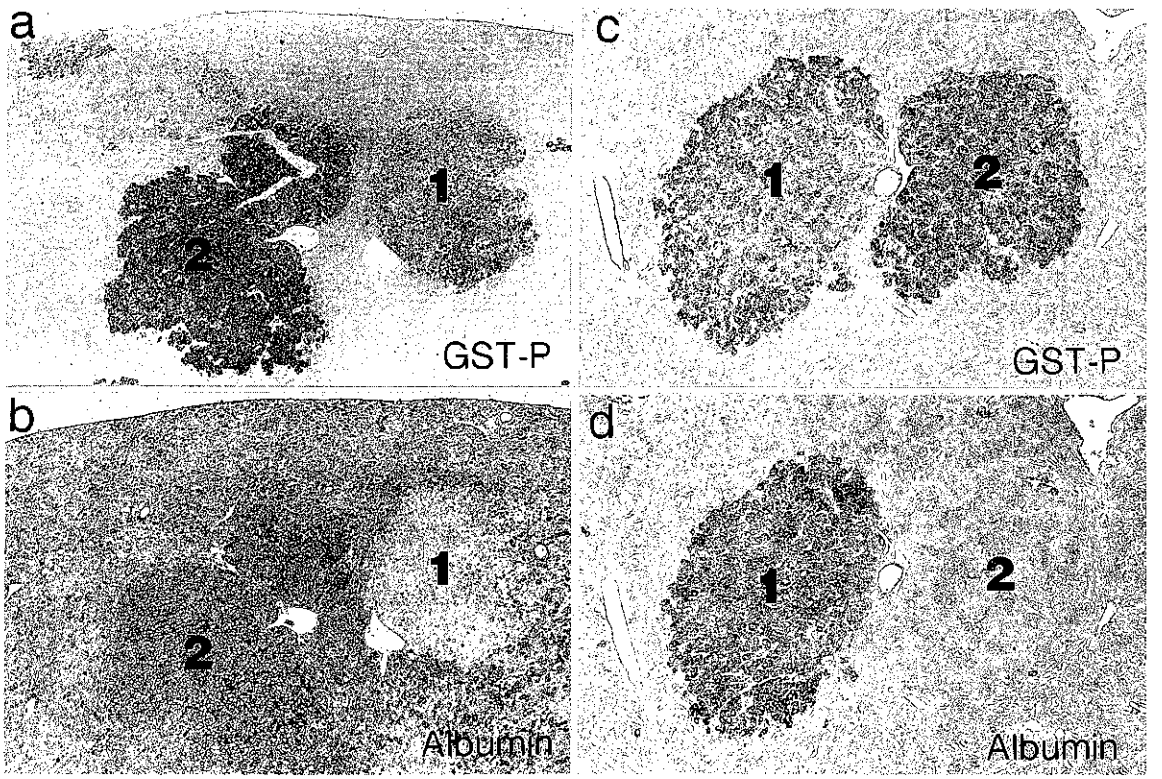

Fig. 4. Immunostaining of serial sections of the livers obtained from two different intrahepatic transplantation models. a, b: model 1 (transplantation of nodule cells from homozygous NAR to livers of heterozygous F1); c, d: model 2 (transplantation of nodule cells from normal rat to liver of homozygous F2). a, c: GST-P immunohistochemistry; b, d: albumin immunohistochemistry. An area containing 2 nodules of different origin was selected and photographed in each model. Nodules designated 1 are considered to be of donor origin, while those designated 2 are considered to be of host origin in each model.

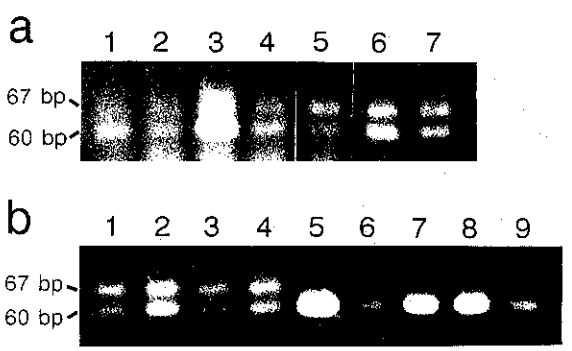

Fig. 5. Ethidium bromide staining of amplified products from tissue isolated from immunostained sections. (a) Model 1. Lanes 1–4: GST-P-positive, albumin-negative nodules (donor origin); lanes 5, 6: GST-P-positive, albumin-positive nodules (host origin); lane 7: GST-P-negative, albumin-positive surrounding liver tissue (host tissue). (b) Model 2. Lanes 1–4: GST-P-positive, albumin-positive nodules (donor origin); lanes 5–7: GST-P-positive, albumin-negative nodules (host origin); lanes 8, 9: GST-P-negative, albumin-negative surrounding liver tissue (host tissue).

In model 1, the weak band of normal rat origin was always detected together with the intense band of NAR origin in the nodules of homozygous NAR origin even though the albumin-negative nodules were carefully collected, free from contamination with the surrounding hepatic tissue. This normal band of host origin is considered to be derived from the stromal cells within the nodules. So, in model 1, differentiation of the donor nodules from the host tissue has to depend on the difference in intensity of the two bands. On the other hand, in model 2, appearance of the band of normal rat origin is direct evidence that the nodule is of donor origin. Thus, model 2 is preferable in the present method. By using the PCR genotyping technique, the carcinogenic process leading from transplanted nodules to hepatocellular carcinomas is under investigation in our laboratory.

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