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(6) てんかん患者に観察される環状20番染色体の解析：遺伝子レベルでのてんかん原因遺伝子の解明

てんかん患者に観察される環状 20 番染色体の解析：遺伝子レベルでのてんかん原因因子の解明

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要旨

1972 年に Uchida らによって第 20 番染色体が環状化したてんかん症例が報告された。このてんかん患者群の核型は、環状型をヘテロに保有したモザイクであることが明らかになっている。環状型の 20 番染色体では、染色体末端部である 20p13 と 20q13.3 領域が結合しているが、現在のところ正確な切断点は不明である。また、本症例の患者では、第 20 番染色体のみが環状化することから、原因は染色体構造を保持する機構の異常ではないと推測される。本研究では、てんかん発症機構を知るために、環状化した 20 番染色体の組み換えによる切断点の位置と周辺領域の異常の有無に注目する。以下の二つの手法により第 20 番染色体の末端領域を解析した. 第 1 の手法は、ヒト DNA のクローン化による物理地図の作成である。第 2 の手法は 蛍光 in situ ハイブリダイゼーション（FISH）法による 解析であり、個々の細胞を観察対象としてシグナルを検出する. 現在までに第 20 番染色体の末端領域に相当する 20p13 と 20q13.3 に位置する 4 つの BAC/PAC コンティグが得られ、16 個の PAC クローンをプローブとして FISH 解析をおこなった。この結果、既知のてんかん原因遺伝子である KCNQ2 や CHRNA4 を含んだ 4 つのコンティグの位置する各領域が、環状染色体にも存在しており環状化の影響を受けていないことが判明した。また、環状染色体にはテロメア配列も観察されることから、環状染色体が、少なくとも一方の末端を完全に保持していることが示された。長腕側の最末端領域に位置する PAC 608I1 と 809E4 は、 サブテロメリック・リピートと考えられる配列を含み、これらのクローンを用いた FISH によって長腕の末端領域が環状染色体に残されていることが示され、長腕部が染色体の環状化とてんかんの発症に関与しないと結論した。

キーワード： てんかん　環状染色体　 ヒト第 20 番染色体

Genetic investigation of the epilepsy associated with ring chromosome 20

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Purpose: Ring chromosome is a rare chromosomal anomaly. An association of ring chromosome 20 with epilepsy was first reported in 1972. The epilepsy syndrome characterized by mental

Retardation, behavioral abnormalities and nonconvulsive status epileptic seizures with unique EEG expression (continuous bilateral high-voltage slow waves with occasional spikes). Ring chromosome 20 is a characteristic of the Epilepsy syndrome and is often observed as the sole cytogenetic abnormality. More than 30 cases have been reported. Most of the epilepsy patients show different rate of ring chromosome mosaicism (1-100%), and the onset age seems to relate to the degree of mosaicism. The locus of ring formation was mapped between 20p13 and 20q13.3 in all cases by cytologic analysis. These characteristics suggest that some kind of malfunction of the chromosome 20 be caused by ring formation. In this respect, CHRNA4 and KCNQ2 genes located on 20q13.3 near the telomere are excellent candidate genes for the epilepsy associated with ring chromosome 20. In order to elucidate the genetic cause of epilepsy with ring chromosome, identification of precise fusion point on the ring chromosome is necessary. Thus, we established PAC/BAC contig map of the critical region to 20p13 (D20S1156-telomere) and 20q13.3 (D20S20-telomere) based on STS content mapping and public databases. Clones from the contigs can be applied to genome analysis on loss of genetic materials in ring chromosome 20.

Methods:

1.End sequencing of PAC clones: Purification of the PAC clone DNA using the QIAGEN Large-Construct Kit. Digesting the DNA with HindIII, the total products were used for sequencing reaction using T7 and sp6 primers by Bigdye terminator sequence kit. After sequencing on ABI377 automated sequencer, homology searches using BLAST2.1 program was carried out to test the presence of repetitive sequences and any other homology. Unique sequences were used as new STS marker to rescreen the library to get addition clones for the presence of STS.

2.PCR analysis: PCR amplification was conducted in 3 step conventional protocol /GeneAmp PCR System 9700. The annealing temperatures varied for different primer sets.

3. Fingerprinting: DNA was digested with BamHI and fractionated on a 0.4% agarose gel in 1 ×TAE buffer

4. Fluorescence in situ hybridization: Probe DNA labeled with biotin and chromosome 20 marker labeled with digoxigenin by nick-translation method.

Slides of metaphase chromosome were hybridized with the labeled probes.

A battery of chromosome 20 probes was used to identify the genetic content of the ring.

Hybridization was performed using 100ng probe per slide at 37℃for 12-16h. Post -hybridization washes were performed in 50% formamide 2×SSC at 42℃ (3 washes, 5 min each) and then in 0.1 ×SSC at 60℃ (3 washes, 5 min each)

Results:

In this study, we have constructed a high-resolution physical map around CHRNA4 and KCNQ2 genes (D20S1156-telomere) and 20q13.3 (D20S20-telomere) regions based on STS content mapping. Total 4 contigs were constructed (Fig.1) and these provided us with a backbone for identifying the precise fusion point of the ring chromosome. Fluorescence in situ hybridization were performed using the PAC clones from those contigs(Fig.2,4) and the telomeric repeat (TTAGGG)n sequence as probes, (Fig.3) along with lymphoid cells from 3 patients with ring chromosome 20 (table 1). All of the hybridization signals were detected in the ring chromosome, indicating that the CHRNA4 and KCNQ2 genes as well as at least one end sequence of the chromosome were intact in these patients. In addition, we confirmed that some kind of fragile terminal structure of chromosome 20 in the patients was generated without a significant deletion.

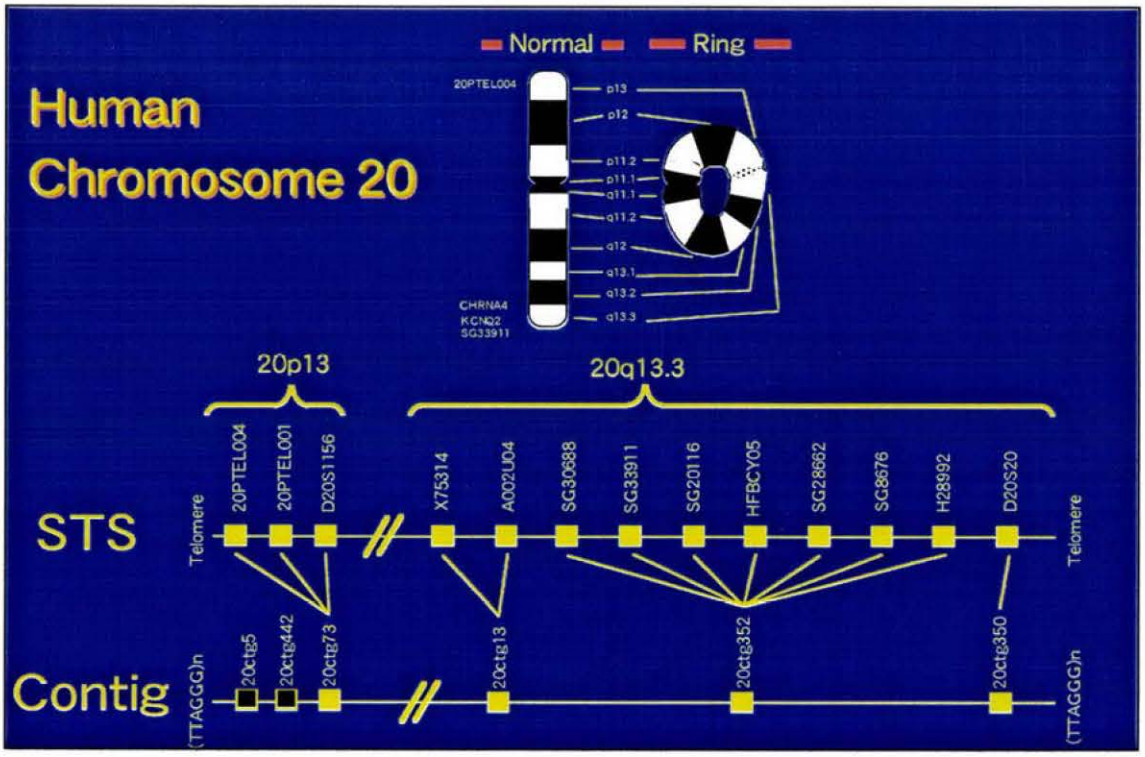


Fig 1. The schematic morphology of human normal and ring chromosome. Three contigs and one contig based on STS mapping and public databases were established in 20q13.3 and 20p13.3, respectively

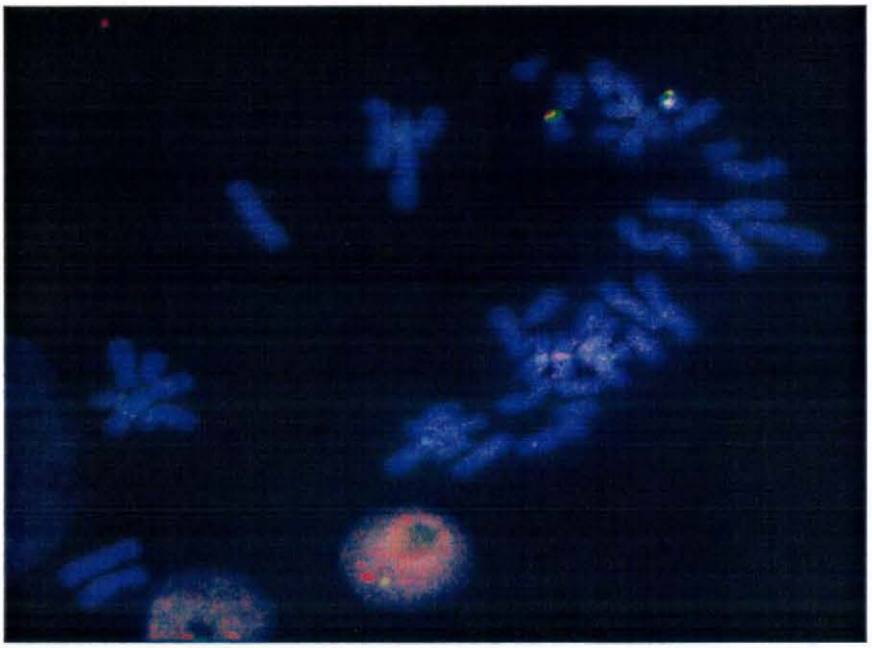


Fig 2. Probe dJ1022E24 (green) is mapped on ctg350. SG33911 content clone dJ610L18 and dJ800N20 were used as specific marker for chromosome 20 (red). FISH signals were present in both normal and ring chromosome 20. Probes mapped on other contigs showed the same signals as this one

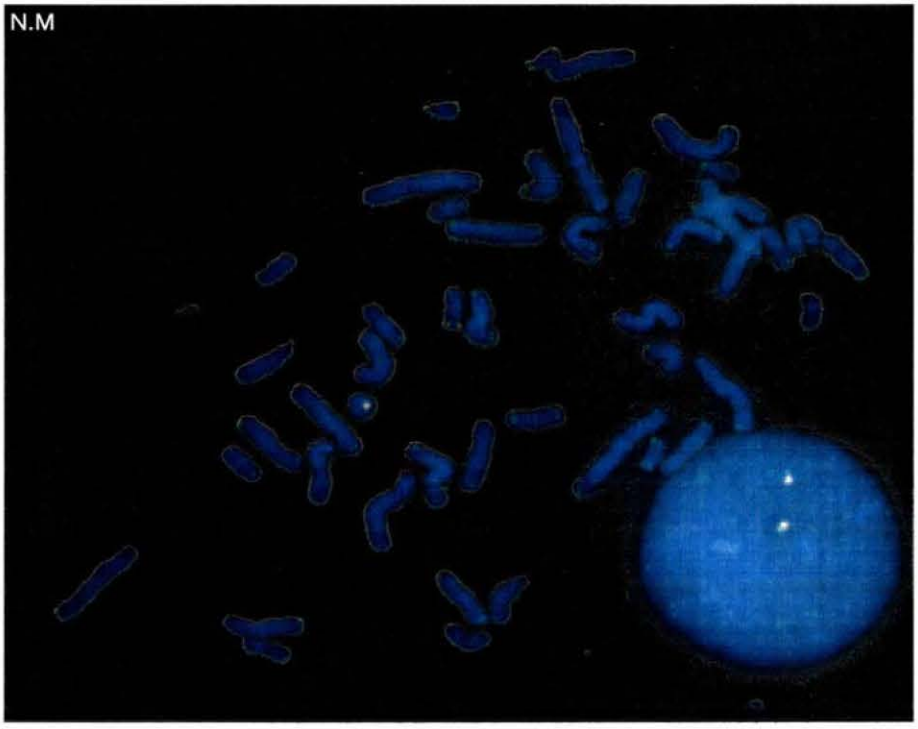


Fig 3. Telomere Repeat (TTAGGG)n (green). Marker dJ800N20 (red). FISH signals were present in both normal and ring chromosome 20. Additional signals appeared in other chromosomes.

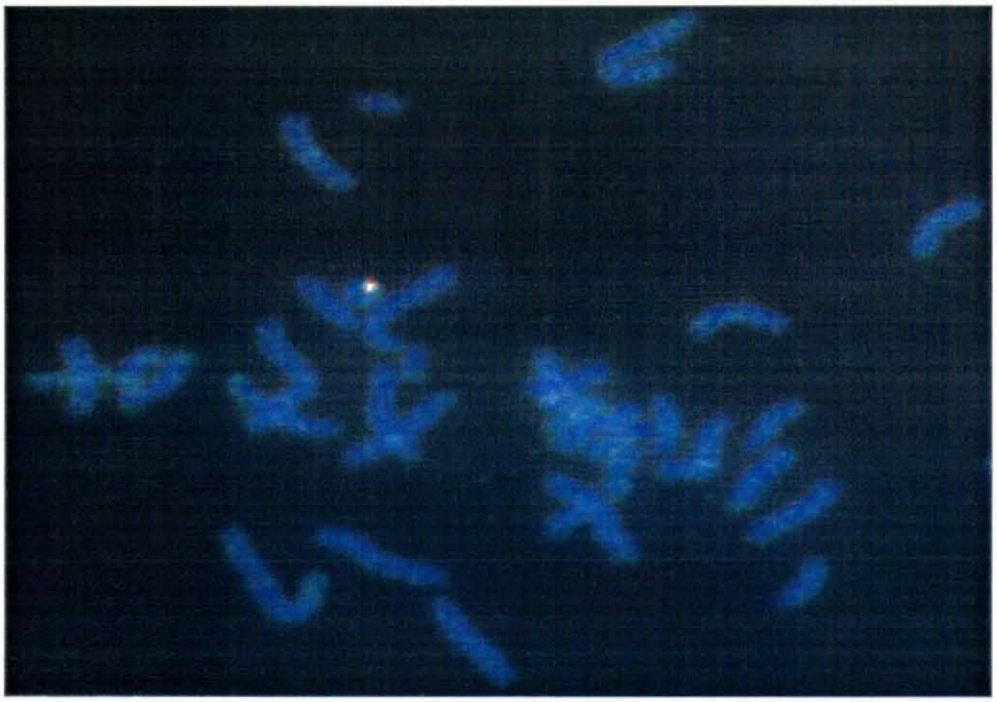
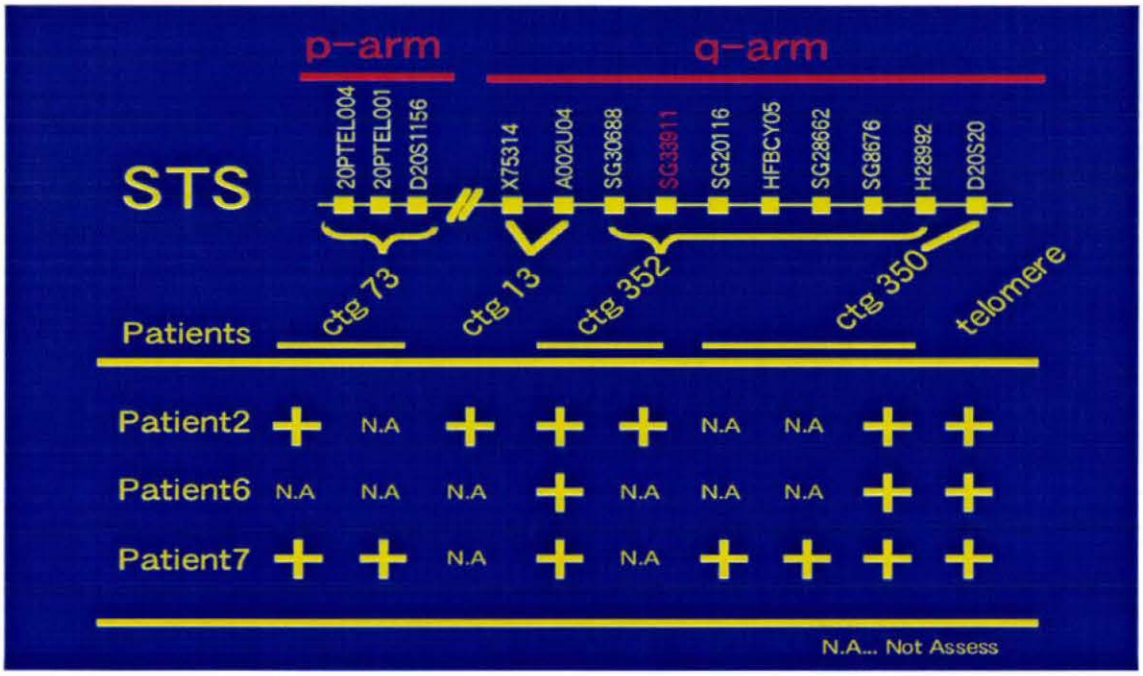


Fig. 4. 7.5kb DNA fragment (green) from the end of ctg350 as probe. FISH signals were also present in the ring chromosome 20. Marker dJ610L18 (red)

Table 1



FISH analysis of ring chromosome (3 patients) Telomere repeat and 16 PAC clones from 3 contigs were used as probes, all signals were present. Observation of positive signal on the ring chromosome 20 is indicated by +

Discussion: It is now well established that ring chromosome 20 are associated with a severe phenotype including non- convulsive status epileptics and a specific EEG in the patients. First the CHRNA4 (neuronal nicotinic acetylcholilne receptor alpha 4 subunit gene) and KCNQ2 (potassium channel gene) located 20q13.3 near the telomere are excellent candidate genes for the epilepsy associated with ring chromosome 20, we constructed a high -resolution map 20q13.3 (D20S20-telomere) regions based on STS content mapping. Use a high-resolution cytogenetic based physical map of 20q13 enabled breakpoints to be assigned to a precise region. 14 PAC clones from the 3 contigs in the end of the long arm and telomeric sequence (TTAGGG) n were used as the probes to detect the content of the ring 20. In our three ring cases, all of the hybridization signals were detected in the ring chromosome, indicating that the CHRNA4 and KCNQ2 genes as well as at least one end sequence of the chromosome were intact in these patients. To gain further insight into the mechanism. We hybridized with probe located in close proximity to the telomeric sequences of the long arm of the chromosome 20, the fluorescence signals were present not only in the long arm of the remaining normal chromosome 20 but also in the ring chromosome. This finding could exclude that the ring chromosome had lost the material from the long arm but now a slight deletion (20p004-telomere) can not be ruled out.

Cote *et al*, 1981 and Kosztolanyi, 1987 have postulated that the ring syndrome is not a consequence of the loss of genetic material but is attributable to the death of cells with secondary aneuploidy caused by instability of the ring chromosome. Pezzolo *et al*, 1993 and Canevini *et al*, 1998 demonstrated that both telomeric and subtelomeric sequences are present in one case of ring chromosome 20.This would further support the hypothesis above. On the other hand, Brandt *et al.* 1993 examined the absence of telomeric sequence in the ring 20 by multicolor PRINS, in this case, Ring chromosomes is thought to be the result of breaks at both ends of the chromosome and subsequent fusion of the open ends. And this mechanism of formation presumes a loss of genetic material. Gene loss from the terminal segment and the disordered equilibrium of the residual genes may be responsible for the occurrence of epileptic seizures.

The diversity of origin of ring 20 will hamper attempts to define the mechanism of ring formation. One possibility for this discrepancy may be that the mechanism was different between the cases reported: when all cells containing ring 20 chromosome before gamete formation, breakage and reunion is involved, while abnormal structure of telomere and a probabilistic process of the terminal fusion of 20 chromosome. The other is consistent with our cases analyzed in this report; all of, which showed mosaics of the cells bearing, ring 20 chromosome and normal cells.

In conclusion, we defined the structure of ring chromosome at molecular level. The ring formation

can occur without loss of the genetic material in the long arm of the chromosome 20. We are now processing identification of the deleted region and screening the difference in genomic structure between normal and ring chromosomes to understand molecular mechanism for chromosome specific rearrangement.

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