We assessed the feasibility of clonality analysis with human androgen receptor gene polymerase chain reaction in terms of the sensitivity and specificity for normal and cancerous colonic tissues taken from fourteen informative cases selected from 22 women with colonic adenocarcinoma. Ten crypts microdissected from each 10-μm-thick cryostat sections and whole tissues were used as samples. DNA was extracted from the samples and amplified with and without prior enzyme digestion. These products were analyzed by capillary electrophoresis for clonality. Of the whole-tissue DNA, none of the normal tissues and seven (50.0%) of the cancerous tissues showed monoclonality. Of the microdissected samples, monoclonality was found in 88.4% (107/121) of normal crypts and 95.9% (117/122) of cancerous crypts. Samples composed of crypts with short and long alleles were found in eight of the 14 normal colonic mucosae, but in none of the cancerous tissues. We concluded that the sensitivity of this method is limited for both whole-tissue DNA and microdissected-tissue DNA, because monoclonality from small samples does not always indicate monoclonality of the entire lesion. The high specificity of this method, however, allows polyclonal results in whole tissues to be confirmed by additional analysis of microdissected tissues.

Key words: clonality analysis, HUMARA, microdissection, colonic mucosa

Introduction

The highly polymorphic trinucleotide repeat of the human androgen receptor gene (HUMARA) makes it the most attractive probe for clonality analysis among the currently available polymorphic DNA probes cloned from X-chromosomal loci.1-4 HUMARA on the X-chromosome has a trinucleotide (CAG) repeat polymorphism in its sequence. The number of this repetition is extremely varied and by analyzing the sequence of the repetition in the 2 strands of X-chromosomes in a woman, in many cases, each paternal and maternal X-chromosome can be identified as having either long or short alleles. Because, one of the X-chromosomes in a woman is inactivated by DNA methylation at an early stage in its development and the restriction enzyme HpaII cannot breakdown methylated DNA, if the makeup of the target HUMARA DNA is disproportionately paternal or maternal, in other words monoclonal, the use of HpaII results in predominantly long alleles or short alleles, revealing the clonality of the target cell population.

The results obtained by using HUMARA, however, are conflicting. Clonality analysis using HUMARA has been effective in establishing the clonality of homogeneous samples such as blood, but its reliability for assessing the clonality of samples from the epithelia and epithelial cancers remains questionable. Some studies using HUMARA have found leukemia,5 cancers of the stomach,6 pancreas,7 and breast8 to be monoclonal, whereas others have shown frequent nonrandom X-inactivation in normal blood, skin, muscle,9 and
nonneoplastic lesions such as endometriosis\textsuperscript{10} and atherosclerosis.\textsuperscript{11} These discrepancies suggest there are limitations to this testing method.

The inconsistent findings are partly due to the anatomical structure of tissues. Samples taken from the epithelia or epithelial tumors are prone to contamination by stromal components during the sampling process.\textsuperscript{12} Using large whole tissue samples thus increases false negative results and decreases sensitivity. To address this issue, studies have been carried out using small samples acquired by laser capture microdissection, a recently introduced sampling technique that can reduce the risk of stromal contamination. This, however, leads to a bias toward monoclonal findings and a decrease in specificity.

The most notable inconsistencies have been found among the studies of colonic lesions, highlighting the difficulty of clonality analysis for colonic mucosa and cancers. Because X inactivation occurs at a relatively early stage of embryonic development, most progeny of single X-inactivated embryonic cells in the adult human are believed to exist in groups of monoclonal cells.\textsuperscript{13,14} These monoclonal cell groups, or patches, have been suspected to influence the evaluation of certain organs such as the colon.\textsuperscript{15,16} A recent study has found the patches in the human colon to be much larger than previously expected, leading to the conclusion that studies using small samples obtained from the colonic mucosa or cancers are biased toward showing monoclonality.\textsuperscript{17}

The purpose of our study was to evaluate the reliability of the HUMARA polymerase chain reaction (PCR) analysis using both whole tissues and microdissected single crypts. To achieve this, we determined the sensitivity and specificity for normal and cancerous colon tissues. This investigation should provide useful additional information about the feasibility of this method for analyzing the clonality of the colonic mucosa and cancer.

Materials and Methods

Patients and samples

Samples were taken from the normal colonic mucosa and cancer of 22 women who underwent colectomy at the Kawaguchi Kogyo General Hospital. Samples were embedded in an optimum cutting temperature (OCT) compound. Three frozen sections were cut from each sample as follows: one 5-μm-thick section for hematoxyline-eosin staining to confirm the orientation of the tissue and two 10-μm-thick sections.

Of the 10-μm-thick sections, one was preserved in a microtube for whole-tissue analysis and the other was placed on PALM membrane slide glasses (P.A.L.M. Mikrolaser Technologie AG, Bernried, Germany) for laser capture microdissection. The ethics committee of the hospital approved the study.

Extraction of DNA and selection of heterozygous cases

DNA was extracted from 10-μm-thick whole-tissue sections by using QiAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The heterozygosity (difference of CAG repeats between the X chromosomes) of the DNA samples from the whole tissues was determined by direct sequencing.

PCR was carried out in a reaction mixture (50 μL) containing 5 μL of template DNA (The DNA extracted was minimal in amount and could not be analyzed), 5 μL of 10×PCR buffer (without MgCl\textsubscript{2}), 3 μL of 25 mM MgCl\textsubscript{2}, 5 μL of dNTP mixture, 5 μL of each HUMARA primer (10 pmol/μL; forward: 5’-GTGCCGCAAGT-GATCCAGAACC, reverse: 5’-TACGATGGCGTTGGG GAGAACC), 0.5 μL of Taq Gold polymerase, and 26.75 μL of DDW. The thermal cycles consisted of an initial cycle of 95°C for 10 minutes. This was followed by 30 cycles of the following steps: 95°C for 45 seconds, 65°C for 45 seconds, and 72°C for 45 seconds. After the last cycle, a final extension step was performed at 72°C for 10 minutes. To detect PCR contamination, a blank control without DNA was always included in every PCR reaction.

These PCR products were purified for DNA sequencing using the MagExtractor (Toyobo, Co., Ltd., Osaka, Japan). The cycle sequencing was carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) as follows: a reaction mixture for cycle sequencing (20 μL) containing 10 μL of purified PCR product, 4 μL of Ready Reaction Premix (Applied Biosystems), 2 μL of 5×BigDye Sequencing Buffer (Applied Biosystems), 0.4 μL of primer (10 pmol/μL; forward: 5’-GTGCCG-GAAGTGATCCAGAACC) and 3.6 μL of DDW. The cycle sequencing consisted of 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes.

To remove excess DyeDeoxy terminator, these cycle sequencing products were purified before DNA sequencing using the CENTRI-SEP COLUMNS (Princeton Separations, Inc., Adelphia, New Jersey, USA). These were dissolved into 20 μL of formamide
(Applied Biosystems), denatured at 95°C for 2 minutes, then immediately cooled on ice. Direct sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Distinctly identical peak patterns were obtained for the two X chromosomes in homozygous cases, but for heterozygous cases, a divergence of the patterns was noted. Because ABI PRISM 310 Genetic Analyzer (Applied Biosystems) detects the difference of two repeats or more, a finding of two CAG repeats or more was determined to indicate that a case was heterozygous, or informative.

Tissue processing and staining
DNA for the HUMARA analysis was extracted from fresh frozen tissues as follows. The 10-μm-thick sections of heterozygous cases were briefly stained with 0.05% toluidine blue for microdissection. Using the P.A.L.M. Robot Microbeam (P.A.L.M. Mikrolaser Technologie AG), 10 crypts were microdissected from each tissue section. DNA was extracted from these crypts using the Pinpoint Slide DNA Isolation System™ (Zymo Research, Orange, California, USA) following the manufacturer’s instructions.

HUMARA PCR analysis
Five microliters of DNA from whole tissues and microdissected tissues were subjected to enzymatic digestion with 5 units of HpaII in the buffer supplied by the manufacturer in a final volume of 6.5 μL. The samples were incubated for 16 hours at 37°C, subjected to heat inactivation at 100°C for 10 minutes, and then cooled on ice.

PCR was performed in a final volume of 50 μL containing 5 μL of template DNA (The DNA extracted was minimal in amount and could not be analyzed), 5 μL of 10X PCR Buffer (without MgCl₂), 3 μL of 25 mM MgCl₂, 5 μL of dNTP mixture, 5 μL of each HUMARA primer (10 pmol/μL; forward IA: 6-FAM labeled 5'-GTGCgCGAAgttgAATCGAGAACC, reverse: 5'-TACGTgggCCTTGGGGAAGAACC), 0.5 μL of Taq Gold polymerase, and 26.75 μL of DDW. Reactions were started with an initial denaturation step at 95°C for 10 minutes. This was followed by 35 cycles of the following steps: 95°C for 45 seconds, 65°C for 45 seconds, and 72°C for 45 seconds. After the last cycle, a final extension step was carried out at 72°C for 10 minutes. All samples were amplified in triplicate.

Clonality analysis using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) was performed as follows: 1 μL of PCR product, 12 μL of deionized formamid (Applied Biosystems), and 0.5 μL of 400HD [ROX] size standard (Applied Biosystems) were mixed and denatured at 95°C for 2 minutes, then immediately cooled on ice. The running conditions were as follows: 15 kV through a capillary with a length of 47 cm and an inner diameter of 50 μm (Applied Biosystems) filled with 310 Genetic Analyzer 10X Buffer with EDTA (Applied Biosystems) and Genetic Analyzer Performance Optimized Polymer 4; POP-4 (Applied Biosystems) at 60°C for 24 minutes. After electrophoresis, peak patterns were automatically analyzed by GeneScan Analysis software (version 3.1.2, Applied Biosystems).

Quantification of clonality
The integration analysis of the peaks allowed the quantification of the data. The ratio of the two peaks in the undigested sample (A/B) was divided by the ratio of the two peaks in the HpaII-digested sample (a/b). A sample was defined to be monoclonal when the corrected ratio indicated that either of the two alleles was present in excess of 50% (corrected ratio > 3) (Figure 1).

![Figure 1. Quantification of clonality.](image-url)

The ratio of the two peaks in the undigested sample (A/B) divided by the ratio of the two peaks in the HpaII-digested sample (a/b). Polyclonal composition with random X-chromosomes would be expected to show ratios equal to or close to 1.0. A sample was defined to be monoclonal when the corrected ratio indicated that either of the two alleles was present in excess of 50% (corrected ratio > 3).
Results

Selection of heterozygous cases for colonic mucosa and cancer

DNA from whole tissue samples of normal colonic mucosae and cancerous tissues from 22 patients were successfully extracted. The number of CAG repeats in DNA extracted from the colonic mucosa samples determined by direct sequencing ranged from 5 to 38 (mean 21.5). Fourteen (63.6%) of the 22 samples showed a difference of two or more CAG repeats between the two X chromosomes (Table, Figure 2) and were determined to be heterozygous. The normal mucosae and cancerous tissues of the 14 patients were used for clonality analysis.

Clonality of whole tissue samples

None of the whole tissue samples taken from the 14 normal mucosae were shown to be of monoclonal origin. Seven (50.0%) of the 14 cancerous tissue samples were determined to be of monoclonal origin. Of these, five were of the short-allele type and two were of the long-allele type.

Clonality of microdissected samples

Each of the crypts that were obtained using laser capture microdissection had an average of 50 cells (Figure 3). Of the crypts obtained from the 14 informative cases, PCR amplification was unsuccessful in 13.6% (19/140) of the normal crypts and 12.9% (18/140) of cancerous crypts.

Analysis of the cell population of the crypts indicated monoclonality in 107 (88.4%) normal crypts and 117 (95.9%) cancerous crypts. Fourteen (10.0%) normal crypts and five (3.6%) cancerous crypts showed a clonality ratio below 3 and were determined to be polyclonal.

Six of the 14 normal mucosae were determined to be monoclonal, because all the crypts in these mucosae had identical alleles (Table). Of these six, four had short alleles and two had long alleles. The remaining eight mucosa samples had a mixture of short and long alleles and were determined to be polyclonal. All 14 cancerous tissue samples were determined to be monoclonal with either a short or long allele, the ratio being 9 to 5 (Figure 4).

Discussion

Our study confirms that in the colonic mucosa, the sensitivity of clonality analysis with HUMARA is limited. Irrespective of the size of the samples, conclusive findings on the monoclonality of an entire lesion cannot be

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<th>Cancerous tissue</th>
<th>Microdissected-tissue DNA from Normal crypts</th>
<th>Number of clonal samples</th>
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*The number of CAG repeats of the human androgen receptor genes from X chromosomes were determined by direct sequencing. The difference in the repeat numbers between two chromosomes are listed in this column; mono-S: Monoclonal with the short allele remaining after digestion; mono-L: Monoclonal with the long allele remaining after digestion; poly: Polyclonal; N.D.: Clonality could not be determined because of PCR failure
obtained by this method of analysis. Large tissue samples are prone to contamination by stromal components or other cells and small samples are affected by the existence of patches. With the specificity being high, results indicating a polyclonal population in whole tissue samples can be interpreted to show the actual polyclonal nature of the epithelial tissues if they are confirmed by the analysis of microdissected samples.

Our analysis of whole tissue samples showed a limited sensitivity of 50% for determining monoclonality. For the 50% of whole tissues which were shown to be monoclonal, we accepted the findings without additional analysis for validation, because the effect of patch size can be ruled out in large samples such as whole tissue samples. Results indicating the presence of a polyclonal population in the remaining 50% of the samples, however, were considered to be inconclusive, because it was not possible to determine whether these result were due to stromal contamination or the actual polyclonal nature of the tissues.

The sensitivity was determined to be 88.4% (107/121) for microdissected normal crypts and 95.9% (117/122) for microdissected cancerous crypts. Because this was based on the findings that normal crypts and cancerous crypts obtained by microdissection are monoclonal, this sensitivity represents sensitivity, not for the entire tissue sample or lesion, but for small groups of cells for which clonality has been determined.

The existence of patches is the primary reason the results cannot be conclusive for the entire lesion. Animal studies have shown that monoclonal groups of up to 450 colonic crypts were distributed over the colonic mucosa, suggesting the analysis of the colonic mucosa to be especially susceptible to the exis-
The presence of patches in human colonic mucosa has been verified using enzyme histochemistry in a study of heterozygous women with one X chromosome coded for inactive mutant G6PD. Reporting these patches to be larger than previously thought, the study concluded that it was virtually impossible to establish the clonality of the entire lesion from the analysis of just a few crypts. Our analysis showed that in six out of 14 normal mucosa samples, all the crypts had the same type of allele, indicating monoclonality and supporting previous studies which have reported the presence of patches. To our knowledge, the present study is the first to show the existence of patches using samples taken from heterozygous women without relying on rare mutations.

The specificity of analysis with HUMARA can be evaluated for normal whole tissues by determining the clonality of a random group of cells considered to be polyclonal. In our study, because all normal whole tissue samples were determined to be polyclonal, we concluded that this confirms a specificity of 100% for whole tissues.

Using microdissected samples can reduce the risk of stromal contamination, but because the patch size does not allow a strictly random group of cells to be obtained, specificity cannot be determined as for whole tissues. The finding that a whole tissue is polyclonal, however, is confirmed if analysis of microdissected samples from that tissue also finds it to be polyclonal. In our study, seven cancerous whole tissue samples were determined to be of monoclonal origin and the remaining seven to be of polyclonal origin, but subsequent analysis of microdissected crypts showed all these samples to be monoclonal, rendering no conclusive results. All normal whole tissue samples were found to be polyclonal and additional analysis of microdissected samples confirmed these findings in eight of these samples.

Although the reason remains unclear, varying degrees of non-random X-chromosome inactivation has been shown in normal tissues. We also found 20% of 100 peripheral blood samples from healthy women (data not shown) to be monoclonal. This confounds the notion that monoclonality indicates malignancy. In our analysis, however, no normal mucosa samples were found to be monoclonal. This consequently facilitated the interpretation of our results.

The analysis of microdissected samples found
10.0% of normal crypts and 3.6% of cancerous crypts to be polyclonal, leading us to suspect that the detection of monoclonal populations was hampered by stromal contamination that occurred during microdissection or by inflammatory cell infiltration in the epithelium. Another explanation for these results is contamination of the microdissected crypts by cells from an adjacent crypt composed of a different type of cell. The possibility that endocrine cells in colonic crypts affected the results should also be considered, because these cells have not been confirmed to be of the same clonal population as epithelial cells.

Sakurazawa et al. assessed the clonality of microdissected crypts taken from aberrant crypt foci in the human colon. Their assessment using HUMARA showed 38% of the crypts to be polyclonal, while the investigation of K-ras mutations showed the crypts to be monoclonal. They concluded that because DNA methylation in aberrant crypt foci is an unstable event which can occur de novo, monoclonal foci can at times appear to be polyclonal. In our study, only a small percentage of crypts were determined to be formed
from polyclonal cell populations: 10% of normal crypts and 3.5% of cancerous crypts. Although we suspect the effect of de novo methylation to be minimal, this point warrants further investigation.

Our method of analysis may shed some light on controversial issues on clonality such as the clonal nature of certain colonic tumors. Tatematsu et al. found in their study on chemical carcinogenesis of chimera mice that cancers with a mixture of two strains of crypts can develop. They surmised this may be a result of a collision of two or more lesions, because such observations were made when clusters of numerous lesions developed in the colon. If, however, sporadic lesions like the ones we studied are shown to be polyclonal, it would confirm that some cancers are indeed polyclonal. Bjerknes et al. studied familial adenomatous polyposis patients using somatic mutation and concluded that adenomas were not clonally pure. It should be noted, however, that their definition of clonality is not clonality as it is generally understood, because their marker contains an acquired mutation. If this adenoma is indeed polyclonal, our analysis should confirm its polyclonal nature.

In conclusion, when using HUMARA for clonality analysis of normal and cancerous colonic tissues, monoclonality can be determined in whole tissues with a 50% sensitivity, but beyond that, even with additional analysis of microdissected samples, conclusive findings on monoclonality cannot be obtained. The high specificity of the analysis, however, guarantees that when a whole-tissue sample is found to be polyclonal and additional analysis of microdissected samples also shows the same, this rules out the possibility of stromal contamination and confirms the polyclonal nature of the tissue. This method of analysis may provide conclusive findings on the possible polyclonal nature of certain cancers and adenomas.

Acknowledgments

The authors thank N. Ando of the Department of Human Pathology, Tokyo Medical and Dental University Graduate School, and Y. Takeichi and N. Katsuta of the Laboratory of Pathology, Kawaguchi Kogyo General Hospital, for technical assistance.

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