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Advances in Brief

Genetic and Functional Analyses Exclude Mortality Factor 4 (*MORF4*) as a Keratinocyte Senescence Gene[1]

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Abstract

Approximately 50% of immortal human keratinocyte lines show loss of heterozygosity of chromosome region 4q33-q34, and the reintroduction of chromosome 4 into one such line, BICR 6, causes proliferation arrest and features of replicative senescence. Recently, a candidate gene, mortality factor 4 (*MORF4*), was identified in this region and sequenced in 21 immortal keratinocyte lines. There were no mutations or deletions, and two of the seven lines that showed loss of heterozygosity at 4q33-q34 were heterozygous for *MORF4* itself. Furthermore, the transfer of a chromosomal segment containing the entire *MORF4* gene did not mimic the senescence effect of chromosome 4 in BICR 6. These results suggest that the inactivation of *MORF4* is not required for human keratinocyte immortality.

Introduction

The immortalization of human keratinocytes is thought to be a permissive condition for the rapid progression of human squamous carcinoma cells (1) and is associated with p53 dysfunction (1, 2), homozygous deletion of the *INK4A* locus (2), $p16^{INK4A}$ dysfunction (2), and high levels of telomerase (2). In addition, immortal human keratinocytes derived from SCC-HN[3] show frequent LOH on chromosome 4 at D4S408/D4S1535 in the 4q33-q34 region (2), and similar regions of loss have been reported in carcinoma of the bladder (3), esophagus (4), and head and neck (5) *in vivo*. Therefore, a gene in this chromosomal region may also be a *bone fide* tumor suppresser *in vivo*. Chromosome 4 causes features of replicative senescence when introduced into several human cell lines (6), including an SCC-HN line, BICR 6, with LOH on 4q.[4] Recently a candidate gene, *MORF4*, a truncated variant of a novel family of putative transcription factors, was reported to map to 4q33-q34.1 and to mimic the senescence effect of chromosome 4 (7). Because this gene mapped close to one of the minimally deleted regions in our panel of cell lines, we tested whether *MORF4* was a likely candidate for the keratinocyte senescence gene. The results show that *MORF4* is unlikely to be this gene.

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[3] The abbreviations used are: SCC-HN, squamous cell carcinoma of the head and neck; *MORF4*, mortality factor 4; LOH, loss of heterozygosity; MRG, *MORF4 related gene*.

[4] N. R. Forsyth, S. A. Fitzsimmons, A. Cuthbert, R. F. Newbold, S. D. Bryce, and E. K. Parkinson, unpublished data.

Materials and Methods

Cell Lines and Culture Methods. The human keratinocyte lines used in this study are listed in Table 1 and were cultured in DMEM containing 20 mM HEPES, antibiotics, 10% v/v fetal bovine serum, and 0.4 μg/ml hydrocortisone (Sigma, Poole, Dorset, United Kingdom). The human fibroblasts were cultured in the same medium with 20% v/v fetal bovine serum and without hydrocortisone.

Microcell-mediated Chromosome Transfer. The generation of the neomycin resistance-tagged human chromosome 4 fragment, F4, and the methods for transferring chromosomes into keratinocytes by microcell-mediated chromosome transfer have been described previously (7, 8).

Measurement of Senescence and Population Doublings. The number of population doublings was measured by recording the cell inputs and cell yields at each passage and using the formula mean population doublings = 3.32 ($\log_{10}$ cell yield − $\log_{10}$ cell input).

The BICR 6 cells that had received an intact copy of chromosome F4 were considered to be immortal when they had completed 50 population doublings because senescence was induced by the intact chromosome 4 within 20 population doublings (6).

Extraction of DNA from Cultured Cells. DNA was extracted from 1×10^6-1×10^7 cells using QIAamp tissue kit (QIAgen Ltd) according to the manufacturer's "blood and body fluid" protocol.

***MORF4* Primer Design, PCR, and Sequencing.** Primers were designed such that the 3′ ends of each oligonucleotide sequence hybridized to positions at which MORF4 differed from the other MRG family sequences (7), this ensured specific amplification and sequencing of *MORF4*. PCR was performed using the primers G1-F: 5′-AAATGGGCTA AATGCCG-TAG-3′ and G1-R: 5′-CACTTTACAG CATATCCCTG-3′, in reactions containing final concentrations of 1 μM each oligonucleotide, 200 μM each dATP, dCTP, dGTP, and TTP, 1.5 mM $MgCl_2$, 1× Reaction buffer II (Perkin-Elmer), 1 unit of Taq polymerase (Perkin-Elmer AmpliTaq), and 100 ng of genomic DNA. Amplification, after a denaturation step for 3 min. at 94°C, used 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, followed by a final extension step of 7 min at 72°C (Perkin-Elmer 9600 thermal cycler). Before sequencing, PCR products were purified using a QIAquick PCR purification kit (QIAgen Ltd.) following the manufacturer's protocol.

MORF4-G1 PCR amplified products were sequenced using six internal primers (three acting in the forward direction and three in the reverse), designed using the same criteria as the PCR primers; S1-F: 5′-CAGTGT-GCTG TATTCAGGAG-3′, S2-F: 5′-ATTGGGATGA TGGGGTTCCG-3′, S3-R: 5′-CAGACCAGAT GTCTTCTTTC C-3′, S4-F: 5′-GCTCTTCTAT CTTCCTGCCG-3′, S5-R: 5′-CCCAACATTA GGTTGAAGTA TT-3′, and S6-R: 5′-CTAACCTCTT CAAAGCACAT CG-3′. Sequencing was performed using 3.2 pmol primer oligonucleotide, 40–80 ng of purified PCR product, and BigDye Terminator cycle sequencing reagents (Perkin-Elmer, Applied Biosystems). Reactions were conducted on a thermal cycler (PCT-100, Genetic Research Instrumentation Ltd.) using 25 cycles of 10 s at 95°C, 5 s at 55°C, and 4 min at 60°C and then run on an ABI 373 sequencer (Applied Biosystems).

Results and Discussion

We have designed *MORF4*-specific PCR primers and established that they are chromosome 4-specific by using a panel of mouse A9 cells, each carrying a single human chromosome. In particular, the primers do not amplify from other human chromosomes to which other members of the MRG family of sequences (7) are reported to map (Fig. 1). We have sequenced, with specific internal primers, a *MORF4* PCR product amplified from genomic DNA that was isolated from normal fibroblasts from many of the SCC-HN patients and one control sample. We also sequenced *MORF4* in 21 immortal keratinocyte lines mainly derived from human SCC-HN (see Table 1). We found no mutations or deletions. Seven of these lines—BICR 6, BICR 18, BICR 19, BICR 22, BICR 31, BICR 78 and BICR 82—show LOH at D4S408/D4S1535, close to the *MORF4* locus at 4q33-q34.1. Any base differences from the *Nhe*I fragment of *MORF4*, which induces senescence in other cell lines after transfection (7), were not cancer-specific and are, therefore, polymorphisms. Examples of these polymorphisms are shown in Fig. 2*a*. The fact that *MORF4* is so polymorphic yields valuable information because, often, different *MORF4* alleles can be distinguished on the basis of sequence. The high frequency of polymorphisms also stresses the need to sequence normal material from the same donor when looking for cancer-specific mutations of this gene. Two lines of the seven that showed LOH at the *MORF4* region, BICR19 and BICR22, were in fact heterozygous for *MORF4* itself; that is, *MORF4* is not in the minimal LOH interval. We were able to show this because of the highly polymorphic nature of *MORF4* (compare BICR19 with BICR16 and BICR31 in Fig. 2*b*). This result also argues against the hypothesis that the loss of one wild-type *MORF4* allele can contribute to immortality through haplo-insufficiency (see Ref. 9). As a direct test of the candidacy of *MORF4*, we introduced a fragment of human chromosome 4, F4 (7), into a keratinocyte line, BICR 6, that is known to senesce after the introduction of an intact copy of human chromosome 4.[4] Three colonies were isolated after G418 selection, and none of them senesced, whereas 11 of 15 of the control HeLa colonies did senesce as reported previously (7). All of the three BICR 6-F4 clones retained the exogenous *MORF4* gene as determined by the polymorphisms at positions 49, 115, 615, 730, and 865 of *MORF4* after sequencing. The sequence of one clone is shown in Fig. 2*c*. The entire sequence was carefully examined for interstitial deletions and point mutations of *MORF4* in these three clones, and none were seen. Even a 1-bp deletion of one *MORF4* allele would be easily recognized as a second sequence on the trace. The retention of the introduced *MORF4* gene at such a high frequency in immortal segregants would not be expected if *MORF4* were the keratinocyte senescence gene on chromosome 4 (10, 11).

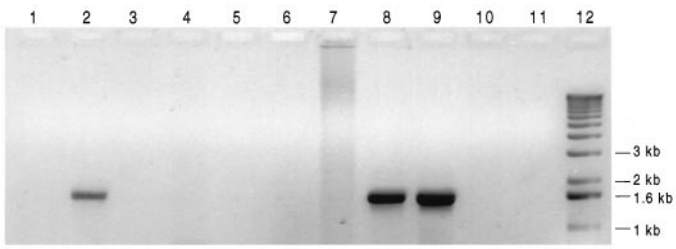

Fig. 1. Amplification of a specific 1526-bp *MORF4* product by PCR only from genomic DNA containing the relevant portion of human chromosome 4. *Lanes 1–7* contain human chromosomes 1, 4, 5, 7, 11, 15, and X, respectively, on an A9 murine background; *Lane 8* contains total human genomic DNA; *Lane 9* contains a chromosome 4 fragment (containing *MORF4*; see Ref. 7) on an A9 murine background; *Lane 10* contains A9 murine genomic DNA,; *Lane 11* contains no DNA (negative control); *Lane 12* contains a 1-kb DNA ladder (Life Technologies, Inc.)

Table 1 *MORF4 sequence alleles*

MORF4 sequence at polymorphic loci in tumor-derived cell lines, normal fibroblast counterparts (*i.e.*, 3F fibroblasts were derived from the same patient as the BICR 3 line, and so forth) and "normal" material used for microcell transfer (F4)/transfection (cloned *Nhe*I fragment; see Ref. 7). Loci base numbering is as used in Fig. 2. Where two bases are indicated at one position, the sequence was found to be heterozygous at this locus; *i.e.*, two different allelic sequences are present in the same cells. The final column indicates lines that are heterozygous and not homonor hemizygous for *MORF4* and the *4q33-q34 LOH* locus in the vicinity of D4S408.

	Base at polymorphic loci							Heterozygous	
	49	108	115	428	615	730	865	*MORF4*	D4S408
Tumor lines									
BICR-3	A/G	T	A/G	G	C/T	A/T	A/G	Yes	Yes
BICR-6	A	T	A	G	C	A	A	No	No
BICR-10	A	T/C	A	G	C	A	A	Yes	ND[a]
BICR-16	G	T	A	G	T	T	G	No	ND
BICR-18	G	T	A	G	T	T	G	No	No
BICR-19	A/G	T/C	A/G	G	C	A/T	A/G	Yes	No
BICR-22	A/G	T	A	G	C/T	A/T	A/G	Yes	No
BICR-31	A	C	A	G	C	A	A	No	No
BICR-56	A/G	T	A	G	C/T	A/T	A/G	Yes	ND
BICR-63	G	T	A/G	G/A	T	T	G	Yes	Yes
BICR-68	G	T	A	G/A	T	T	G	Yes	Yes
BICR-78	A	C	A	G	C	A	A	No	No
BICR-82	A	T	A	G	C	A	A	No	No
SCC-4	A	T/C	A	G	C	A	A	Yes	ND
SCC-9	G	T	A/G	G	T	T	G	Yes	ND
SCC-12	A/G	T/C	A/G	G	C/T	A/T	A/G	Yes	ND
SCC-13	A/G	T/C	A	G	C	A	A	Yes	ND
SCC-15	A/G	T	A/G	G	C	A/T	A/G	Yes	ND
SCC-25	A	T	A	G	C	A	A	No	ND
SV-HFK	A	T	A	G	C	A	A	No	ND
DOK	A	C	A	G	C	A	A	No	ND
Fibroblasts									
3F	A/G	T	A/G	G	C/T	A/T	A/G	Yes	N/A
6F	A	T/C	A	G	C	A	A	Yes	N/A
18F	G	T	A	G/A	T	T	G	Yes	N/A
22F	A/G	T	A	G	C/T	A/T	A/G	Yes	N/A
31F	A/G	T/C	A	G	C/T	A/T	A/G	Yes	N/A
56F	A/G	T	A	G	C/T	A/T	A/G	Yes	N/A
63F	G	T	A/G	G/A	T	T	G	Yes	N/A
68F	G	T	A	G/A	T	T	G	Yes	N/A
78F	A	T/C	A	G	C	A	A	Yes	N/A
82F	A	T/C	A	G	C	A	A	Yes	N/A
59F	G	T	A/G	G	T	T	G	Yes	N/A
Normals									
F4	G	T	G	G	T	T	G	N/A	N/A
*Nhe*I clone	A	T	A	G	C	A	A	N/A	N/A

[a] ND, not done; N/A, not appropriate.

Our previous work (2, 6, 7·[4]) has indicated that there may be a novel senescence gene on human chromosome 4, and the work of others (3–5) suggests that the same chromosomal region contains a genetic suppressor of certain human carcinomas. The evidence presented above suggests that *MORF4* is unlikely to be either the gene that causes senescence in SCC-HN cells or the suppresser of SCC-HN tumors *in vivo* that maps to the same chromosomal region (5). The keratinocyte senescence gene could still map to 4q33-q34, but other regions of 4q also display high frequencies of LOH both in SCC-HN lines (2) and in *in vivo* tumors (5). In particular, the 4q24-q26 region has been implicated in the pathogenesis of SCC-HN (5) and in keratinocyte immortalization (2), but current evidence suggests that this region carries a gene that suppresses tumor development without causing replicative senescence (12). Further work is, therefore, required to map and identify the chromosome 4 gene involved in keratinocyte senescence and immortalization.

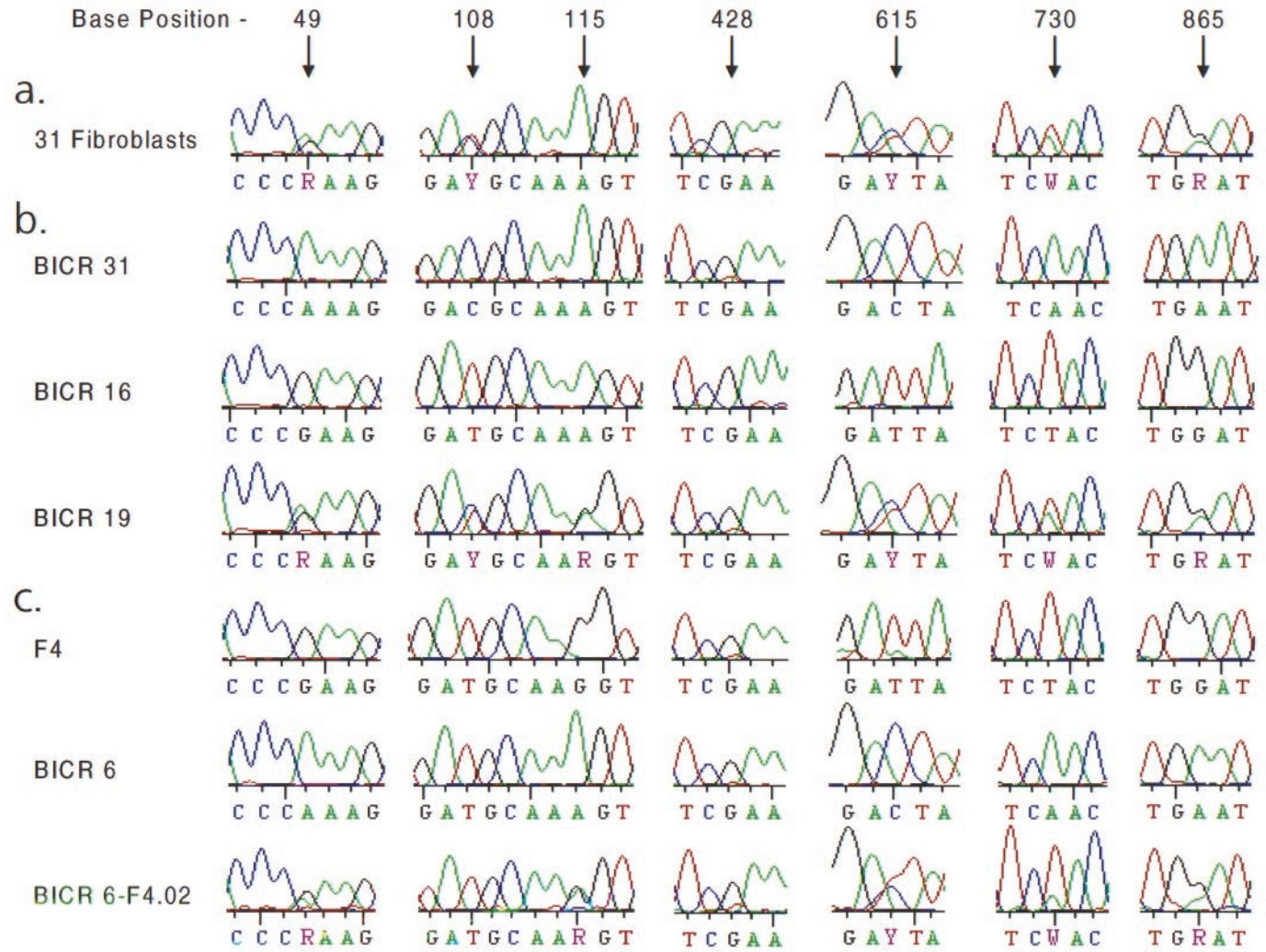

Fig. 2. Sequence traces at polymorphic loci in the *MORF4* gene, bases (*arrows*) are numbered such that position +1 corresponds to the first base after the 5′ flanking repeat in the *MORF4* sequence (7). Bases are labeled R when both A and G peaks are present, Y when C and T peaks are present, and W when A and T peaks are present. *a*, heterozygous sequence showing multiple polymorphic loci in normal fibroblast (from the same patient from which the BICR 31 SCC-HN line was derived) DNA. *b*, sequences homozygous for different sequence alleles of *MORF4* (*BICR 31* and *BICR 16*) and a sequence heterozygous for *MORF4* (*BICR 19*) in SCC-HN tumor-derived cell lines. *c*, sequence of exogenous *MORF4*-containing chromosome fragment F4, endogenous *MORF4* from BICR6 and a BICR 6-chromosome F4 hybrid (*F4.02*) showing retention of the exogenous *MORF4* allele.

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