Mechanisms of disease

Genetic heterogeneity of single disseminated tumour cells in minimal residual cancer

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Summary

Background Because cancer patients with small tumours often relapse despite local and systemic treatment, we investigated the genetic variation of the precursors of distant metastasis at the stage of minimal residual disease. Disseminated tumour cells can be detected by epithelial markers in mesenchymal tissues and represent targets for adjuvant therapies.

Methods We screened 525 bone-marrow, blood, and lymph-node samples from 474 patients with breast, prostate, and gastrointestinal cancers for single disseminated cancer cells by immunocytochemistry with epithelial-specific markers. 71 (14%) of the samples contained two or more tumour cells whose genomic organisation we studied by single cell genomic hybridisation. In addition, we tested whether *TP53* was mutated. Hierarchical clustering algorithms were used to determine the degree of clonal relatedness of sister cells that were isolated from individual patients.

Findings Irrespective of cancer type, we saw an unexpectedly high genetic divergence in minimal residual cancer, particularly at the level of chromosomal imbalances. Although few disseminated cells harboured *TP53* mutations at this stage of disease, we also saw microheterogeneity of the *TP53* genotype. The genetic heterogeneity was strikingly reduced with the emergence of clinically evident metastasis.

Interpretation Although the heterogeneity of primary tumours has long been known, we show here that early disseminated cancer cells are genomically very unstable as well. Selection of clonally expanding cells leading to metastasis seems to occur after dissemination has taken place. Therefore, adjuvant therapies are confronted with an extremely large reservoir of variant cells from which resistant tumour cells can be selected.

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Introduction

The refinement of molecular and cellular techniques, such as laser microdissection combined with PCR and cytogenetic analyses, have revealed that almost every human primary carcinoma has huge genetic heterogeneity.[1–6] From a clinical point of view, the genetic heterogeneity of primary tumours has no consequences for local treatment because timely surgery will remove the entire locally grown tumour. However, with regard to systemic therapy in an adjuvant setting, whether the target population is genetically homogeneous or heterogeneous is a relevant question. Adjuvant treatments currently dominate clinical efforts to prevent metastatic disease[7–10] because overall mortality from solid cancers has only slightly decreased in recent years.[11] Occult tumour-cell dissemination is the most likely reason for these disappointing results, since it can lead to lethal relapses months or years after supposedly curative surgery.

This latent stage of the disease is characterised by the spread of tumour cells undetected by standard diagnostic techniques, and their persistence has been termed MINIMAL RESIDUAL CANCER or MINIMAL RESIDUAL DISEASE, which should be distinguished from clinically evident metastasis. Single disseminated tumour cells derived from epithelial tissues can now be detected in minimal residual cancer with antibodies against cytokeratins in bone marrow and blood[12] and against the epithelial cell adhesion molecule (EpCAM) in lymph nodes.[13] However, these tumour cells are only found at extremely low frequencies of 1 in 10^5–10^6 normal nucleated cells in about 30% of carcinoma patients with small locally restricted tumours.[14] Although their presence is strongly associated with an increased risk of metastasis for all major types of cancer,[15–21] the biology of these cells has largely remained unexplored.[22] This lack of knowledge hampers the design of effective adjuvant therapies for the elimination of disseminated cancer cells in minimal residual cancer.[23]

Because invasion and dissemination are generally believed to be late events in tumour progression and are restricted to specialised clones of the primary tumour, we aimed to analyse directly the composition of the disseminated cell population in patients with minimal residual cancer to find out whether they form a homogeneous and thus possibly selected cell population.

Methods

Patients

We needed to detect at least two of the extremely rare disseminated tumour cells per patient. Therefore, we screened more than 500 bone-marrow, lymph-node, and blood samples from 474 patients. For comparison, we isolated single cells from cancer patients with clinically manifest metastasis, the intention being to contrast cells which disseminated early from small primary tumours with those apparently metastasising from established metastases.

GLOSSARY

COMPARATIVE GENOMIC HYBRIDISATION
Genome-wide screening technique to detect chromosomal gains and losses by hybridisation of labelled tumour DNA in comparison with differently labelled DNA from normal cells onto metaphase spreads of healthy donors.

GENETIC INSTABILITY
Term for the increased mutation rate in tumour cells, used to explain the huge number of mutations seen in human carcinomas.

METAPHASE
Phase of the cell cycle in which the chromatin is highly condensed, enabling identification of the individual chromosomes on the basis of characteristic banding patterns.

MINIMAL RESIDUAL DISEASE/MINIMAL RESIDUAL CANCER
Occult tumour left behind after so-called curative surgical resection of the primary carcinoma, potentially leading to relapse months or years after surgery. The material correlates of minimal residual cancer are single disseminated carcinoma cells that can be detected in mesenchymal organs such as bone marrow, blood, or lymph nodes by histogenetic markers. Their presence in bone marrow and lymph nodes is associated with shortened overall survival for all major types of cancer.

SINGLE-STRANDED CONFORMATIONAL POLYMORPHISM ANALYSIS
Method by which to detect point mutations of DNA sequences by conformational alterations that result from nucleotide sequence changes. The altered conformation of a single-stranded DNA fragment is visualised in a non-denaturing polyacrylamide gel because it leads to different migration behaviour.

TP53
The P53 protein has been designated the "guardian of the genome"; its "tumour suppressor" gene *TP53* is mutated in about 50% of malignant tumours.

All carcinoma patients had bone-marrow aspiration or lymph-node sampling, after informed consent was obtained, as part of clinical routine diagnosis or within clinical studies approved by the local ethics committees. Patients' illnesses were staged postoperatively according to the standard tumour node metastasis (TNM) classification of the Union Internationale Contre le Cancre (UICC).

Detection and isolation of single disseminated tumour cells

The procedure for bone-marrow preparation has been described previously[20] and was done at the clinical centres from which the samples were obtained. After isolation of mononuclear cells, 2×10^6 cells were placed on positively charged glass slides (Micromet, Munich, Germany) at a density of 500 000 cells per 227 mm^2, leaving enough space between individual cells for subsequent isolation. After sedimentation, slides were dried and shipped to the Institut für Immunologie, Munich.

From each patient, $1–2\times10^6$ bone-marrow or blood cells were stained either by use of the monoclonal antibody A45-B/B3 (Micromet, Munich, Germany) against cytokeratin 8, 18, and 19, or with the monoclonal antibody CK2 (Roche, Mannheim, Germany) directed against cytokeratin 18 by means of the alkaline phosphatase/anti-alkaline phosphatase technique.[17,20] Cell suspensions from lymph-node samples were prepared with a Medimachine (Dako, Hamburg, Germany), prepared as described for bone-marrow cells above, and were then stained by use of the EpCAM antibody BerEP4 (Dako).[18] Alkaline phosphatase was developed with 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue tetrazolium (BCIP/NBT; BioRad, Munich, Germany) as substrate, slides were covered with phosphate-buffered saline under a cover glass, and assessed by bright-field microscopy. An identical number of cells served as a control for staining with mouse IgG1 Kappa (MOPC-21) without known binding specificity. After removal of the cover glass, positive cells were isolated from the slide with a micromanipulator and placed in phosphate-buffered saline containing 0·5% non-ionic detergent (Igepal; Sigma, Munich, Germany). Before transfer to the PCR reaction tube, all isolated single cells were placed on a fresh slide and examined to ensure that no contaminating cells were co-isolated.

Global amplification and comparative genomic hybridisation

We next analysed these samples to find out how disseminated cancer cells that stem from one individual tumour are genetically related. All isolated cells were separately investigated by a recently developed method for whole-genome amplification of a single cell.[24] This technique, known as global amplification, is based on the generation of an *Mse*I representation of the genome, which is obtained after digestion with the restriction enzyme *Mse*I, adaptor ligation, and PCR amplification by a single primer. The amplicons obtained via this procedure can be used for COMPARATIVE GENOMIC HYBRIDISATION, because this type of amplification reliably preserves the numerical ratios of a given karyotype,[24] or for numerous gene-specific PCRs. In a minor modification to the published protocol, the adaptor sequences were formed with the primers 5′-AGTGGGA TTCCTGCTGTCAGT-3′ and 5′-TAACTGACAG ddC-3′. Tumour-cell DNA was digoxigenin-labelled and control DNA biotin-labelled. In short, DNA from the single tumour cells and normal control DNA were simultaneously hybridised to normal human chromosomes in METAPHASE and were developed with two different fluorochromes. For each chromosomal region, fluorescent intensities were compared and evaluated with the Leica software package Q-CGH. Losses and gains were regarded as significant when the ratio of tumour DNA to control DNA was below 0·75 and higher than 1·25, respectively.

	Primer pair
Exon 4 I	
Forward	5′-GCTCTTTTCACCCATCTACAG-3′
Reverse	5′-GAAGGGACAGAAGATGACAG-3′
Exon 4 II	
Forward	5′-CTGCACCAGCAGCTCCTAC-3′
Reverse	5′-GAAGTCTCATGGAAGCCAG-3′
Exon 5	
Forward	5′-TCACTTGTGCCCTGACTTTCA-3′
Reverse	5′-TCTCCAGCCCCAGCTGCT-3′
Exon 6	
Forward	5′-TTCCTCACTGATTGCTCTTAG-3′
Reverse	5′-GACCCCAGTTGCAAACCAG-3′
Exon 7	
Forward	5′-GCGCACTGGCCTCATCTTG-3′
Reverse	5′-CACAGCAGGCCAGTGTGCA-3′
Exon 8	
Forward	5′-AGGACCTGATTTCCTTACTGC-3′
Reverse	5′-GAATCTGAGGCATAACTGCAC-3′
Exon 9	
Forward	5′-GTGCAGTTATGCCTCAGATTC-3′
Reverse	5′-GAGGTCCCAAGACTTAGTAC-3′

Table 1: **Primers used in *TP53* sequence analysis**

TP53 *sequence analysis*

We screened all disseminated tumour cells identified for mutations in the *TP53* gene—ie, one of the most commonly mutated genes in human cancer.[25] Analysis of the DNA sequence of *TP53* was restricted to exons 4–9 because more than 97% of mutations lie within this region.[26]

TP53 PCR was done with an MJ PTC 200 Thermocycler, and comprised: one cycle of 94°C for 120 s, 58°C for 30 s, and 72°C for 120 s; 14 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 20 s; 24 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s; and a final extension step of 72°C for 120 s. The primers used are shown in table 1. All forward primers were labelled with fluorescein isothiocyanate at the 5′ end. SINGLE-STRANDED CONFORMATIONAL POLYMORPHISM ANALYSIS was done at 6°C on a 15% acrylamide/bisacrylamide (29:1) gel containing 1·5× tris-borate-EDTA in the presence and absence of 5% glycerol for 8 h (with the exception of exons 8 and 9, which were run for 16 h). The gels were analysed on a FluorImager SI (Molecular Dynamics, Freiburg, Germany). All bands showing a different migration behaviour were sequenced (Sequiserve, Vaterstetten, Germany).

Analysis of dendrogram

We transformed the comparative genomic hybridisation profiles of all single cells isolated into a table, with each chromosomal arm being classified as deleted (–1), amplified (+1), or not-affected (0). To determine the relatedness of the individual cells, we applied a hierarchical clustering algorithm (available from http://rana.lbl.gov/EisenSoftware.htm, accessed Aug 19, 2002).[27] The algorithm organises the comparative genomic hybridisation data on the basis of overall similarity in their genomic aberration patterns. These relationships are summarised in a dendrogram, in which the pattern and length of the branches reflect the relatedness of the samples.

For quantitative analysis, we measured the length of the branches between two cells. To adjust for the different composition of the groups, we normalised all lengths by setting the maximum distance between the baseline and the first branching of each dendrogram to 100. We then applied a two-sided Student's *t* test for statistical comparison between the two groups.

Role of the funding source

None of the funding sources was involved in the study design, data collection, data analysis, data interpretation, or writing of the report.

Results

We isolated cytokeratin-positive cells from bone marrow or blood, and EpCAM-positive cells from lymph nodes, after screening 525 samples from cancers of the breast (n=304), gastrointestinal tract (n=115), and prostate (n=106). Examples of such cells are shown in figure 1. 142 (27%) of the samples contained disseminated tumour cells, and 71 samples (14%) contained two or more cells (figure 1).

After global amplification and comparative genomic hybridisation, all control cells displayed normal profiles, suggesting that these techniques had not introduced any aberrations themselves. In 29 of the 71 samples containing two or more disseminated tumour cells, representing mostly breast cancer patients, we noted that not all cells contained genomic changes detectable by comparative genomic hybridisation. Although isolated as single cells (figure 1), we could not decide whether these cytokeratin-positive or EpCAM-positive cells without changes in their comparative genomic hybridisation profile were indeed tumour cells, whose mutations are below the resolution limit of comparative genomic hybridisation (10–20 Mb),[28] or staining artefacts. Therefore cells with normal comparative genomic hybridisation profiles were excluded from further analysis.

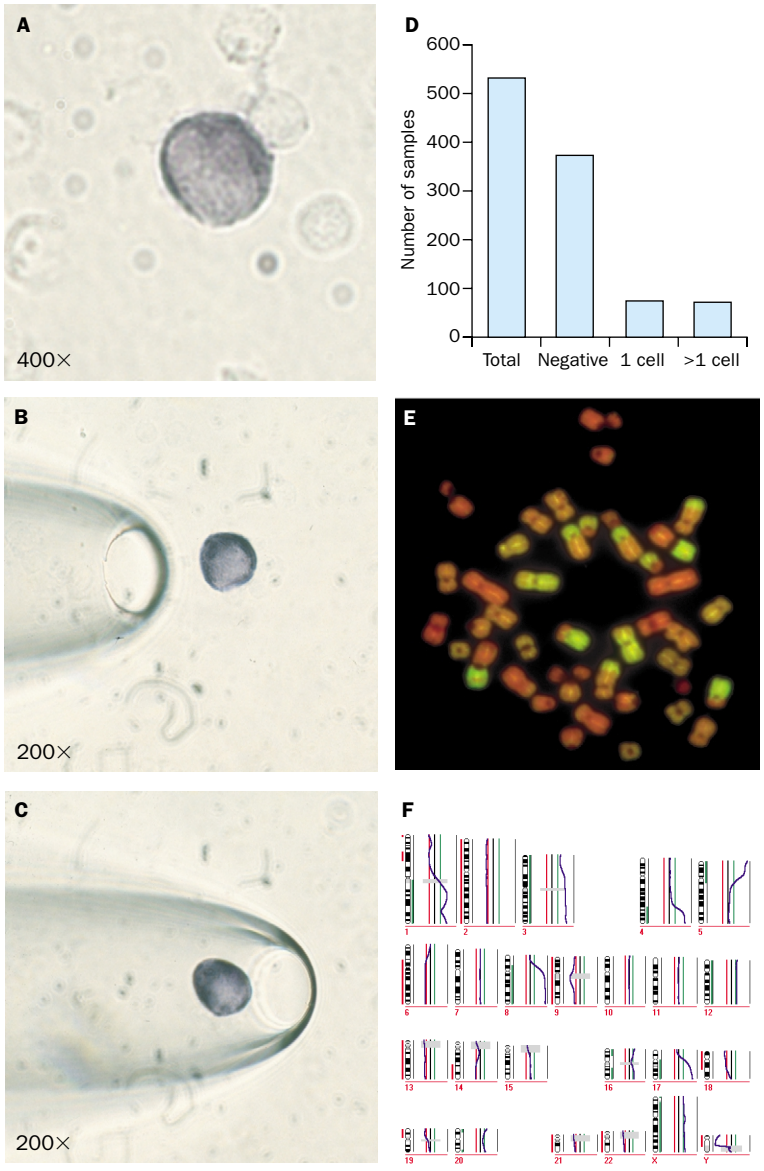

Figure 1: **Cytokeratin-positive and EpCAM-positive cells, and results of comparative genomic hybridisation**

A: Cytokeratin-positive cell between unstained bone-marrow cells. B: Single cytokeratin-positive cell after removal of non-stained cells before isolation by micromanipulator. C: Cytokeratin-postive cell detached from slide within capillary of micromanipulator. D: Number of samples screened for presence of disseminated tumour cells and that contained no, one, or more than one cytokeratin-positive or EpCAM-positive cell. E/F: Normal metaphase hybridised with labelled tumour cell DNA (green) and control DNA (red), and resulting comparative genomic hybridisation profile (F). Blue line represents calculated ratio of fluorescent intensities, which is normally between the red (ie, loss) or green (ie, gain) thresholds for significance. Red or green bars next to chromosome ideogram indicate significant gains or losses.

The remaining 42 bone-marrow samples contained 115 cells displaying aberrations. These cells were subjected to cluster analysis of comparative genomic hybridisation profiles. Cells that were isolated from minimal residual cancer patients (UICC stage M0) and from patients with overt metastatic disease (UICC stage M1) were analysed separately. The dendrogram of cells isolated from patients with metastatic disease revealed a distinctly closer relation among the cells of the individual patient than among the cells from patients with minimal residual cancer (figure 2). This difference in heterogeneity deserves even more attention with regard to the significantly higher number of aberrations per cell seen in the patients with advanced metastatic disease (M0=6·7 *vs* M1=11·8; p<0·0001). Figure 2 shows that the cells of some patients diverge to such a degree that they are assigned to rather distantly related branches of the dendrogram (eg, P-004-2 and P-004-1, M-007-1 and M-007-3).

Figure 2 also depicts all genomic aberrations of disseminated tumour cells from two patients. On average, the distance (in relative units) between two cells isolated from a patient with minimal residual cancer is 57, whereas two cells of a patient with metastatic disease are 27 apart (p=0·0016). Therefore, disseminated tumour cells from patients with manifest metastases (M1) are much more homogeneous than cells from minimal residual cancer.

Additionally, we analysed single disseminated cells over time in six patients—three in stage M0, two in M1, and one prostate cancer patient who presented first in M0 and 1 year later in M1. All aspirates had been taken at various times, 6 or more months apart. From four additional patients we had samples from two different organs—bone marrow and lymph node—from which we could detect single disseminated cells and compare their genomic aberrations. The lymph-node samples had been taken during primary surgery and therefore all these patients were in stage M0.

Although cells isolated from patients with metastatic disease had generally very similar genomic changes at the different sampling points, cells isolated from patients with minimal residual cancer were generally very heterogeneous over time (figure 3). Moreover, the cells that were derived from an individual patient but isolated from different organs shared almost no alteration (figure 3). The first bone-marrow sample from patient P-105 had been taken when he presented with increasing concentrations of prostate-specific antigen after radical prostatectomy for prostate cancer—ie, during so-called biochemical relapse. 1 year later, when the patient had clinically overt metastasis, three cytokeratin-positive cells could be isolated that shared several genomic aberrations. However, figure 3 shows that gain of chromosome 8q was already present 1 year previously. These observations suggest firstly that the cells that spread throughout the body early on are genetically very unstable, and that the selection acting on this very heterogeneous cell population during systemic cancer progression finally results in the expansion of a tumour cell with a relatively stable genome. Additionally, the genetic data support the interpretation that minimal residual cancer might be divided into "active" (eg, biochemical relapse in prostate cancer) and "dormant", in which an advantageous mutation is acquired shortly before a highly aggressive metastatic clone appears.

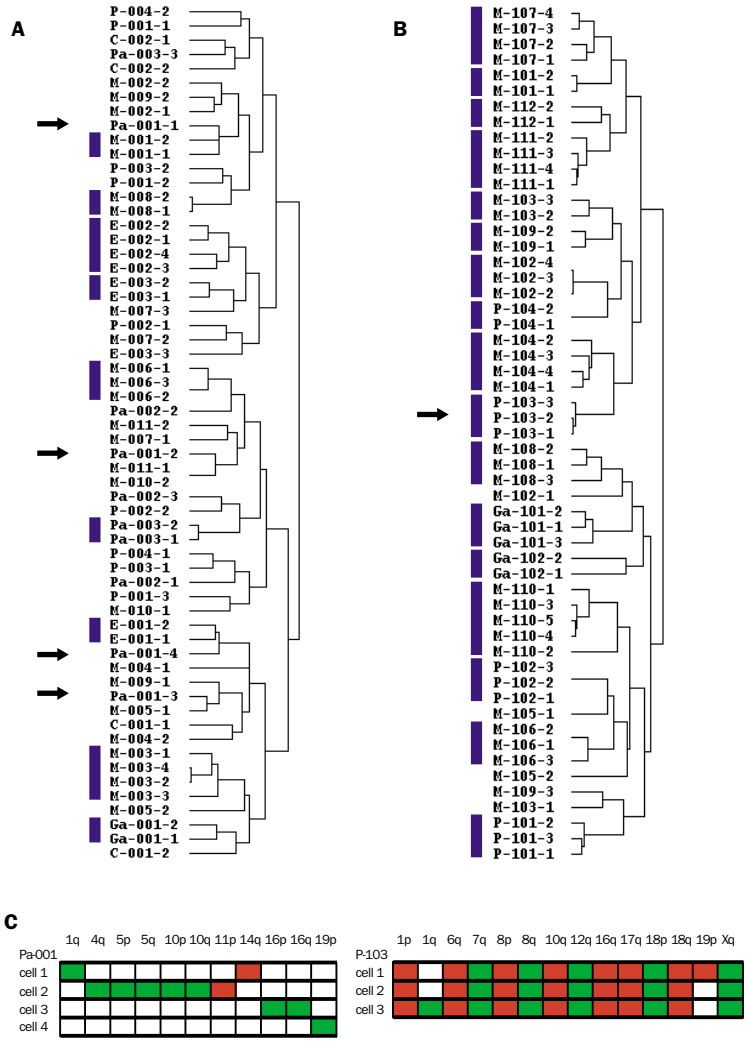

Figure 2: **Dendrogram of minimal residual cancer cells (A) and disseminated cells from patients with manifest metastasis (B), and examples of comparative genomic hybridisation results (C)**

A/B: Blue bars indicate cells from one patient grouped within one branch of the dendrogram. Tumour types are indicated by first letter of identifier (M=breast; P=prostate; E=oesophagus; C=colon; Pa=pancreas; Ga=gastric cancer) and patients by three-digit numbers (001–011 for stage M0 and 101–112 for patients in stage M1). Single-digit numbers indicate individual cells from one patient. C: Comparative genomic hybridisation aberrations of cells 1–4 of patient Pa-001 and cells 1–3 of patient P-103 exemplify clustering: cells from Pa-001 do not share a single aberration and are consequently placed far away from each other, whereas cells from patient P-103 that share almost all aberrations are clustered together. Arrows indicate cells from Pa-001 and P-103 in the dendrogram.

Because of the huge range of chromosome aberrations possible in single cells, be it in the early stage of minimal residual cancer or in the late stage with clinically manifest metastasis, we wondered whether we could also see a kind of microheterogeneity in single cells disseminated from one individual tumour by focusing on defined gene loci. To this end, we concentrated on the *TP53* gene as one of the most commonly mutated genes in human cancer,[25] and screened all cells identified for *TP53* mutations.

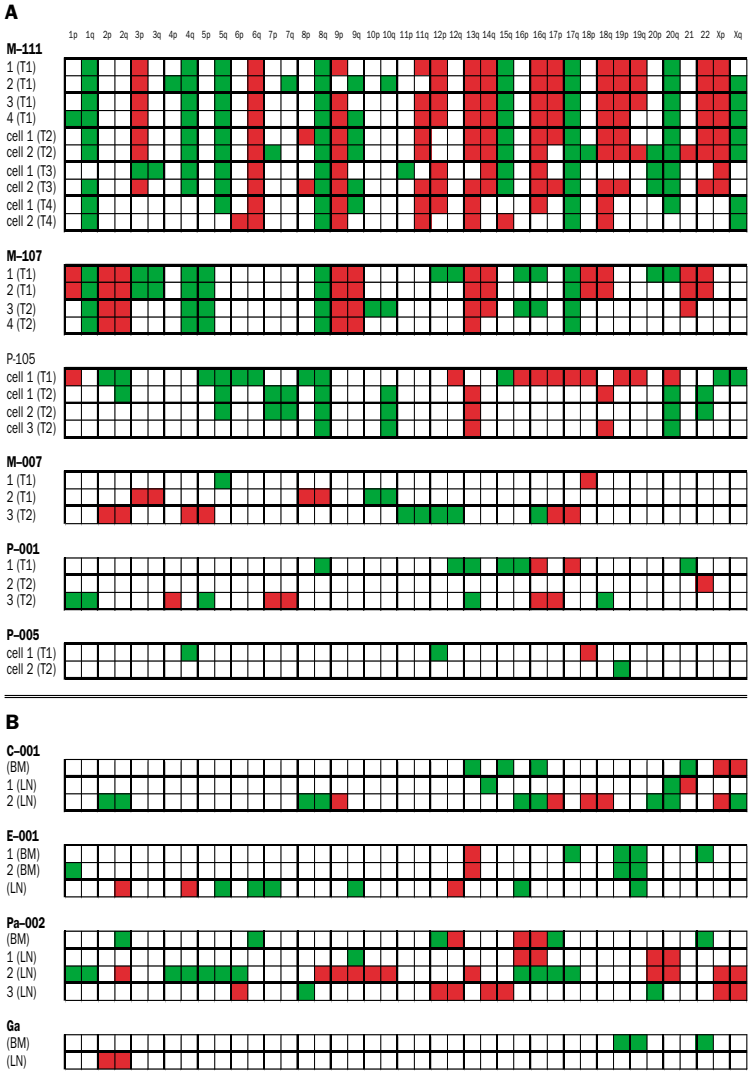

Figure 3: **Genomic analysis of disseminated tumour cells isolated at different time points (A) or from two different organs (B) of the same individual patient**
Green fields designate genomic gains, red fields genomic losses for the respective chromosome arm. Patient identifiers as in figure 2. Timepoint and organs from which cells were isolated are given in parentheses. From patient M-111, only cells from T1 had been used for cluster analysis. P-105 was not included in dendrogram because disease progressed from M0 (T1) to M1 between bone-marrow analysis (T2). In B we added cells that were not included in cluster analysis because only one cell was isolated from one organ. These cells are designated only by organ from which they were isolated. T=timepoint; BM=cell isolated from bone marrow; LN=cell isolated from lymph node.

To verify the sensitivity of the approach, we analysed 12 cell lines each carrying a defined *TP53* mutation, and showed that under the established conditions all specific mutations were reliably identified. To assess the probability of artificially introduced mutations during the amplification, identical experimental conditions were applied to 46 normal single cells isolated from bone-marrow or peripheral blood.

Single-stranded conformational polymorphism analysis (figure 4) showed that none of the 322 (seven primer pairs×46 cells) PCR products obtained from the single control cells showed any change in migration behaviour, which indicates that PCR-induced mutations in the tumour cells would be an extremely rare event. By contrast, 19 of 115 tumour cells contained point mutations that all led to aminoacid exchanges, truncations, or mutations in the splice-acceptor site; two cells showed a homozygous deletion of *TP53* alleles (table 2). Therefore, most cells (82%) did not harbour *TP53* mutations.

However, the tumour cells from three of four patients were heterogeneous for *TP53* genotype at the stage of minimal residual disease. In clinically evident metastatic disease, this heterogeneity was seen only in breast cancers, in which *TP53* mutations seem to occur rather late in tumour progression. Because there is increasing evidence that *TP53* mutations can confer loss of function as well as gain of function,[29] the finding of multiple *TP53* genotypes suggests a divergent functional consequence that probably increases the heterogeneity of the minimal residual cancer cell population. The multiple mutations, and hence the heterogeneity of *TP53* mutations, would have been missed if pooled DNA of the isolated cells had been analysed.

Discussion

Our data provide new insight into the evolution and progression of systemic cancer. Strikingly, minimal residual cancer cells are highly heterogeneous irrespective of whether they reside within the same compartment or within different homing sites, or whether they are isolated on repeated bone-marrow aspirations. We believe our conclusion is valid for the following reasons. First, cytokeratin and EpCAM are highly specific markers for tumour cells in bone marrow and blood and lymph nodes, respectively, and have been validated in numerous clinical trials.[15,17,18,20,21] Second, isolation of stained cells from single-cell suspensions by mechanical micromanipulation not only prevents contamination by normal cells but also excludes the possibility of DNA changes introduced by high-energy laser microdissection into a single-cell genome. Third, our single-cell comparative genomic hybridisation protocol was extensively controlled, and fourth, thresholds of significance in the comparative genomic hybridisation analysis were set to levels such that contamination by a single normal cell would blur the genomic aberrations of the tumour cell. Thus all cells from which our conclusions are drawn represent individual tumour cells.

Whether the observed heterogeneity is a consequence of the clonal diversification of the primary tumour or GENETIC INSTABILITY continuing after the cells spread throughout the body is unclear. Thus far, we could not detect any change common to a high proportion of early-disseminated tumour cells. Even the gene most frequently mutated in human cancer—ie, *TP53*—seems to be very rarely mutated in the minimal residual cancer cell population. However, this finding does not yet allow strong conclusions to be drawn because minimal residual cancer cells of different tumour types can harbour *TP53* mutations at different frequencies.[25] In some cases, disseminated cancer cells might have to acquire *TP53* mutations for clonal expansion into metastasis.[30] For most tumours, however, *TP53* mutations do not seem to occur early in oncogenesis.

However, the heterogeneity of minimal residual cancer cells does not prove a truly polyclonal origin of the disseminated cells. Because of the limited resolution of metaphase comparative genomic hybridisation, and the fact that possibly epigenetic changes such as aberrant

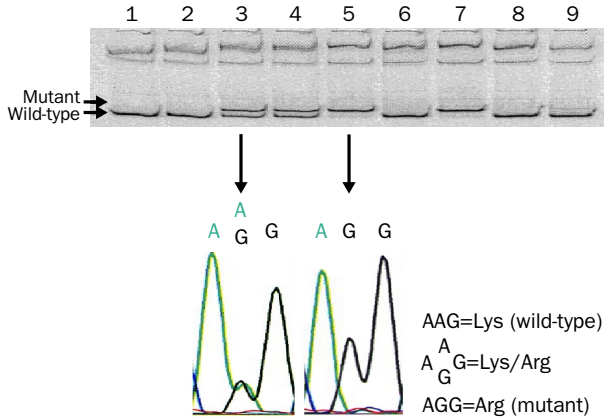

Figure 4: ***TP53* single-stranded conformational polymorphism analysis (exon 5) of five single tumour cells (lanes 3–7) and four single control cells (lanes 1, 2, 8, 9)**

methylation patterns might precede chromosomal aberrations,[31] further studies should be done to resolve this question. Nonetheless, clinically evident metastasis is apparently preceded by a genetic diversification present in early-disseminated tumour cells, from which certain, more aggressive, and "fitter" genotypes are selected. Since the mere finding of disseminated tumour cells in stage M0 patients of all major tumour types indicates a bad prognosis,[15–21] we can conclude that this cell population comprises the precursors of metastasis that are eventually selected from a large reservoir of genetic variant cells. Support for this reasoning comes from the remarkable homogeneity seen in M1 cells, thus showing that not all disseminated cells at the M0 stage are likely to grow into a metastasis. Nevertheless, the extreme heterogeneity in minimal residual cancer puts a caveat on all adjuvant therapies that rely on a single agent and on all animal models working with transplanted, cloned tumour cell lines.

So far, our analysis of minimal residual cancer points to a cell population in which no two individual tumour cells are identical, strongly supporting the concept of genetic instability.[32,33] Because about 1–10 million disseminated tumour cells form the occult cancer cell population in minimal residual cancer, the finding of several *TP53* genotypes or even mutations in the few detected and analysed cells from an individual tumour points to a high number of variants even for such critical mutations. Consequently, despite the growing enthusiasm about the insights into the development of cancer and new selective drugs and treatments, future therapies and the establishment of adequate experimental models are confronted with this extreme tumour-cell heterogeneity, the most tormenting problem in cancer research.

Stage	Cell number	Genotype	Mutation
M0	E-002-1	Exon 5 not amplified	..
	E-002-2	Mutated/wild-type (exon 5)	Codon 175 (Arg→His)
		Mutated/wild-type (exon 8)	Codon 296 (His→Tyr)
	E-002-3	Mutated (exon 5)	Codon 175 (Arg→His)
	E-002-4	Mutated/wild-type (exon 5)	Codon 175 (Arg→His)
M0	E-004-1	Mutated (exon 6)	Codon 221 (Glu→Lys)
	E-004-2	Exon 6 not amplified	..
	E-004-3	Wild-type	..
M0	M-007-1	Mutated/wild-type (exon 8)	Codon 277 (Cys→Phe)
	M-007-2	Wild-type (exon 8)	..
	M-007-3	Wild-type (exon 8)	..
M0	Pa-002-1	Wild-type (exon 8)	..
	Pa-002-2	Wild-type (exon 8)	..
	Pa-002-3	Mutated (exon 8)	Codon 292 (Lys→Ile)
M0	Pa-003-1	Mutated (intron 5)	Splice G→A
	Pa-003-2	Mutated (intron 5)	Splice G→A
M1	M-110-1	Mutated/wild-type (exon 5)	Codon 132 (Lys→Arg)
	M-110-2	Mutated/wild-type (exon 5)	Codon 132 (Lys→Arg)
	M-110-3	Mutated (exon 5)	Codon 132 (Lys→Arg)
	M-110-4	Wild-type (exon 5)	..
		Mutated/mutated (exon 6)	Codon 200 (Asn→Thr), codon 202 (Arg→Leu)
	M-110-5	Mutated (exon 5)	Codon 132 (Lys→Arg)
M1	M-111-1	Homozygous deletion	..
	M-111-2	Homozygous deletion	..
	M-111-3	Wild-type	..
	M-111-4	Mutated/wild-type (exon 5)	Codon 153 (Pro→Ser), codon 159 (Ala→Ser)
M1	P-101-1	Mutated/wild-type (exon 7)	Codon 245 (Gly→Asp)
	P-101-2	Mutated (exon 7)	Codon 245 (Gly→Asp)
	P-101-3	Mutated (exon 7)	Codon 245 (Gly→Asp)
M1	P-104-1	Mutated (exon 5)	Codon 136 stop
	P-104-2	Mutated (exon 5)	Codon 136 stop
	P-104-3	Exon 5 not amplified	..

Table 2: ***TP53* mutations in disseminated cancer cells**

Contributors

C Klein contributed to data analysis and manuscript preparation; Thomas Blankenstein did the *TP53* analysis; O Schmidt-Kittler did the comparative genomic hybridisation of breast cancer cells; M Petronio did the comparative genomic hybridisation of tumour cells from colon, gastric, and pancreatic cancers; N Stoecklein did the comparative genomic hybridisation of oesophageal cancer cells; B Polzer did the comparative genomic hybridisation of prostate cancer cells; and G Riethmüller contributed to manuscript preparation.

Conflict of interest statement

Christoph Klein is a consultant for Micromet AG and is an inventor of the patent on single-cell DNA amplification held by Micromet AG; Oleg Schmidt-Kittler is also an inventor of the patent on single-cell DNA amplification held by Micromet AG; Gert Riethmüller is a founder and partner of Micromet AG.

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Uses of error

Medical history

Jan Faergemann

A decade ago, a 54-year-old man was referred to me because his face, hands, and nails had started to turn grey during the previous 7 summers. Clinically, the grey-black discolouration of his skin was a clear-cut case of argyria. I asked him specifically if he was taking any medication, particularly anything containing silver salts, but his answer was no. The patient had played the horn since childhood, and currently played in the Gothenburg Symphony Orchestra. The mouthpiece of the horn is coated in silver, and I thought that the uptake of silver in the skin could come from this unusual source. Metallic silver is not usually the course of argyria; salts such as silver nitrate are much more common. I did a biopsy and ruled out other causes of dark discolouration such as haemochromatosis. I explained my diagnosis to the patient and told him that unfortunately, the discolouration of his skin would remain for a very long time. My only suggestion was that he could protect himself against the silver by inserting a plastic coating in the mouthpiece of the horn. I also told him that he should be careful with the sun because tanning of the skin would increase the darkness of his discolouration.

I forgot about the patient until I saw him again approximately 6 months later at the end of the summer. He was coming back because the colour of his skin, in sun-exposed areas, was now intense grey-black and because it was difficult for him to use the plastic coating of his mouthpiece. Again I asked him if he was taking any kind of medication and this time he told me that he had been taking an antitussive every night for over 10 years. This drug contained 3% silver nitrate. Of course this was the obvious explanation for the argyria. He could stop taking this unnecessary medication, and go back to playing his horn without a useless plastic insert. Even though his skin would remain permanently discoloured, it would no longer progress, and he now knew the correct cause of his disease.

This patient reminded me of the difficulties in obtaining a correct medical history. One should always maintain a healthy index of suspicion if another explanation seems more likely than the patient's account.

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