Published in final edited form as:

Int J Cancer. 2009 February 1; 124(3): 589–599. doi:10.1002/ijc.23949.

Genomic events associated with progression of pleural malignant mesothelioma

Sergey V. Ivanov^{1,§}, Jeremy Miller², Robert Lucito³, Chunlao Tang³, Alla V. Ivanova¹, Jianming Pei⁴, Michele Carbone⁵, Christina Cruz¹, Amanda Beck¹, Craig Webb², Daisuke Nonaka⁶, Joseph R. Testa⁴, and Harvey I. Pass¹

¹Thoracic Surgery Laboratory, Cardiothoracic Surgery Department, NYU Langone Medical Center, NY 10016

²Laboratory of Tumor Metastasis and Angiogenesis, Van Andel Institute, Grand Rapids, MI 49503

³Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724

⁴Human Genetics Program, Fox Chase Cancer Center, Philadelphia, PA19111

⁵Cancer Research Center of Hawaii, Honolulu, HI 96813

⁶Department of Pathology, NYU Langone Medical Center, NY 10016

Abstract

Pleural malignant mesothelioma (MM) is an aggressive cancer with a very long latency and a very short median survival. Little is known about the genetic events that trigger MM and their relation to poor outcome. The goal of our study was to characterize major genomic gains and losses associated with MM origin and progression and assess their clinical significance. We performed Representative Oligonucleotide Microarray Analysis (ROMA) on DNA isolated from tumors of 22 patients who recurred at variable interval with the disease after surgery. The total number of copy number alterations (CNA) and frequent imbalances for patients with short time (<12 months from surgery) and long time to recurrence were recorded and mapped using the Analysis of Copy Errors (ACE) algorithm. We report a profound increase in CNA in the short-time recurrence group with most chromosomes affected, which can be explained by chromosomal instability associated with MM. Deletions in chromosomes 22q12.2, 19q13.32, and 17p13.1 appeared to be the most frequent events (55-74%) shared between MM patients followed by deletions in 1p, 9p, 9q, 4p, 3p and gains in 5p, 18q, 8q, and 17q (23-55%). Deletions in 9p21.3 encompassing *CDKN2A/ARF* and *CDKN2B* were characterized as specific for the short-term recurrence group. Analysis of the minimal common areas of frequent gains and losses identified candidate genes that may be involved in different stages of MM: *OSM* (22q12.2), *FUS1* and *PL6* (3p21.3), *DNAJA1* (9p21.1), and *CDH2* (18q11.2-q12.3). Imbalances seen by ROMA were confirmed by Affymetrix genome analysis in a subset of samples.

Keywords

mesothelioma; ROMA; CGH; copy number alterations; tumor suppressors; oncogenes

[§]Corresponding author: Sergey V. Ivanov, tel. (212)-263-0409, fax (212)-263-2011, e-mail: Sergey.Ivanov@med.nyu.edu .

Here we report the first use of ROMA with CGH-Explorer software for genome-wide detection of chromosomal deletions and gains in mesothelioma. The results of the study show that disease progression is associated with chromosomal instability and identify recurrent genomic events that correlate with prognosis.

INTRODUCTION

Malignant mesothelioma (MM) of the pleura is an aggressive cancer often associated with exposure to asbestos. MM has a very long median latency of 43.6 years¹ and a very short median survival of approximately 11 months². MM cytogenetics reveals a highly variable karyotype with multiple rearrangements. The most frequent events observed in MM are deletions in 1p21-22, 3p21, 4, 6q14-25, 9p21, 13q, 14q, 15q15, 17p13, and 22³. Monosomy 22 is the most frequent numerical change. A more detailed insight into chromosomal rearrangements associated with cancer became possible with the advent of the comparative genomic hybridization (CGH) fluorescent technology that permits high resolution analysis of DNA copy number variation^{4, 5}. CGH analysis of cell lines and tumor specimens derived from MM patients has confirmed previous karyotypic abnormalities and has revealed other genomic alterations, such as losses of 15q11.1-15, 14q24.2-qter, and 13q12-14, as well as gains on 5p^{6, 7}. Recently, the development of microarray-based versions of CGH has greatly increased resolution and throughput, enabling the analysis of genomic imbalances of less than 100 kb in size⁸. Representational oligonucleotide microarray analysis (ROMA) is one such technology, which uses complexity reducing representations to increase probe signal to noise for the comparison of tumor to normal cells⁹. In this study, we report the results of ROMA analysis performed on 22 tumors from MM patients who had surgery for the disease, with the goal to define chromosomal regions affected during disease progression.

MATERIALS AND METHODS

Mesothelioma patients and specimens

Patients having resection of mesothelioma by the senior author (HIP) while he was at Wayne State University, Detroit Michigan from 1998-2005 gave written consent for procurement of tumor and normal peritoneum, as approved by the Wayne State University Institutional Review Board (D1420: Collection of Serum and Tissue Samples for Patients with Biopsy-Prove or Suspected Malignant Disease). These specimens upon transfer to NYU School of Medicine through a cooperative materials transfer agreement were approved for discovery of prognostic and early detection biomarkers by the NYU School of Medicine Institutional Review Board. 27 snap-frozen specimens (22 tumors and 5 unaffected peritonea harvested from the peritoneum of patients undergoing extrapleural pneumonectomy) derived from 22 patients with MM were used to produce 27 DNA samples for ROMA analysis. 4 out of 5 normal samples were tumor-matched. As seen in Table 1, 9 patients showed disease longer than 12 months after surgery, and 13 progressed within 1 year of surgery.

DNA isolation and analysis

DNA from snap-frozen tissues was isolated with QIAamp mini-kit (QIAGEN, Valencia, CA) and assayed for concentration and quality using Victor3 microplate reader (PerkinElmer, Waltham, MA) and agarose electrophoresis. *Bgl*II genomic representations, and Cy3-dCTP (reference DNA) or Cy5-dCTP (DNA of interest) incorporation, hybridization, and washing conditions were done as described recently¹⁰. Hybridizations were carried out on arrays bearing 85,000 oligonucleotides (NimbleGen, Systems, Madison, WI). Slides were scanned with an AxonGenePix 4000B scanner (Axon Instruments, Union City, CA). Enzymes used in the study were *Bgl*II and T4 DNA ligase (New England Biolabs, Ipswich, MA). Primers were supplied by Sigma Genosys (St. Louis, MO). Cot1 DNA and tRNA were obtained from Invitrogen (Carlsbad, CA). The Megaprime labeling kit, Cy3-conjugated dCTP, and Cy5-conjugated dCTP were purchased from Amersham Biosciences (GE Healthcare, Piscataway, NJ). Taq polymerase Mastermix was supplied by

Eppendorf (Westbury, NY). NimbleGen photoprint arrays were synthesized by NimbleGen Systems Inc. (Madison, WI).

ROMA

Arrays were described previously 10. In brief, the arrays are based on representational techniques and, thus, all oligonucleotides map to BglII fragments that are within the representations size range of 200-1000 bp. The array was designed to NCBI Build 30 and the coordinates have been updated to NCBI Build 35. Sample preparation, BglII representations, has been described previously 11. DNA was labeled as described with minor changes 11. Procedures for hybridization were followed as reported previously 12. Arrays were scanned with an Axon GenePix 4000B scanner set at a pixel size of 5 μ m. GenePix Pro 4.0 software was used to quantify the intensity for the arrays. Array data were imported into S-PLUS for further analysis. Measured intensities without background subtraction were used to calculate ratios. Data were normalized using an intensity-based lowest curve fitting algorithm.

ROMA data processing

Signal intensity ratios were normalized against standard ROMA control (normal DNA specimen isolated from human skin fibroblasts) for each of 27 probes (including 5 normal pleura specimens) used in the study. The data file was imported into CGH-Explorer v. 2.55 available from <http://www.ifi.uio.no/forskning/grupper/bioinf/Papers/CGH/>, log2-transformed, meancentered, and used for visualization and statistical analysis. Statistical evaluation was done using the ACE (analysis of copy errors) algorithm available with the software 13. To control the error of multiple comparison without losing discovery power, the expected proportion of false discoveries was estimated using the false discovery rate (FDR) 14.

Affymetrix genome analysis

In the validation studies, Affymetrix GeneChip® Human Mapping 250K Nsp arrays containing ~262,000 SNPs and Genome-Wide Human SNP Arrays 6.0 containing more than 906,600 single nucleotide polymorphisms (SNPs) and more than 946,000 probes for the detection of copy number variation were used as the hybridization targets. The array probe datasheets can be found at the web sites http://www.affymetrix.com/support/technical/datasheets/500k_datasheet.pdf and http://www.affymetrix.com/support/technical/datasheets/genomewide_snp6_datasheet.pdf, respectively. Briefly, total genomic DNA (250-500 ng) from each test sample was digested with *NspI* or *StyI* restriction enzyme and ligated to an adapter that recognizes cohesive 4-basepair (bp) overhangs. A generic primer that recognizes the adapter sequence was used to amplify the adapter-ligated DNA fragments. PCR conditions were optimized to preferentially amplify fragments in the 200- to 1,100-bp size range. After purification using Microcon Ultracel filters, the amplified DNA was fragmented with DNase I, biotin-labeled, and hybridized to the GeneChip according to the manufacturer's recommendations. For the 250K NspI arrays, the intensities of probe hybridization were analyzed using Affymetrix GCOS 1.4 software, and the genotyping was performed using GTYPE 4.1 with Dynamic Model Mapping Analysis by default settings at 0.33 for both homozygote and heterozygote call thresholds. Copy number analyses were carried out using Affymetrix Chromosome Copy Number Analysis Tool (CNAT) version 4.0.1. For the 6.0 arrays, the genotyping and copy number analyses were performed using Affymetrix Genotyping Console 2.1 with Birdseed genotype calling algorithm and Chromosome Copy Number Analysis Tool (CNAT) version 5.

Gene expression profiling

RNA from 22 tumor specimens used in our ROMA study were isolated and analyzed for differential expression as described 15 using the Affymetrix U133A platform. For independent validation of candidate genes with regard to their expression, we used an additional, completely different set of MM (n=30) and normal pleura (n=7) specimens for comparative gene expression study on the U133 2.0 Plus platform.

RT-PCR analysis

RT-PCR premixes were prepared using a Super Script One-Step RT-PCR kit (Invitrogen, Carlsbad, CA). Primers for RT-PCR assessment of gene expression were designed as described 16. Conditions for the reaction were as follows: 50°C — 30 min., 35 cycles of (94°C — 15s, 56°C- 15s, 72°C — 1 min.), 72°C — 5 min. Oligonucleotide sequences for RT-PCR were designed to ensure RNA-specific synthesis across introns: *OSM*: 5'-CTTGGAAGAAGCTGCAGATGG-3', 5'-AGCCTCTAACTCCCTAGCTTC-3', *FUS1*: 5'-AACTCCCAGGCTCAATCAAG-3', 5'-CAGACTCTGCCACGACATC-3', *PL6*: 5'-GCTTGACTCGGGTACAGAAC-3', -5'-CTATGGCGCTGGTAGAAGC-3', *CDH2*: 5'-CTGCTTCAGGCGTCTGTAG-3', 5'-ATTGCCTTCCATGTCTGTAG-3', *DNAJA1*: 5'-CGGGTTCGGCTACAAAAGAG-3', 5'-GATGACGATGGTTCGGTTGTC-3', *MKI67*: 5'-GATGTGGAAGTTCTGCCTACG-3', 5'-GCGGTTGCTCCTTCACTG-3'. RT-PCR with primers to invariantly expressed *PPIA* (5'-TCTGAGCACTGGAGAGAAAGG-3', 5'-GGAAAACATGGGAACCCAAAGG-3') was used as a loading control. RT-PCR was performed on a set of matched normal-tumor specimens from 8 MM patients. Band intensities were measured using Kodak 4000 Image Station. Repeated experiments showed consistency of our RT-PCR analysis.

RESULTS

Copy number alterations in patients with long-and short-term recurrences

MM patients used for ROMA were represented by two groups: the one with short-term recurrence after surgery (less than 12 months, STR) and the other one with longer time to relapse (LTR). Five normal tissue specimens were assembled into the third group (NORM). The total number of events for each specimen was calculated using the ACE algorithm available from the CGH-Explorer package 13. Table 1 shows demographics of the patients as well as total CNA counts (gains and losses estimated by the total amount of differentially hybridized probes) for each tumor specimen with the false discovery rate of 0.023 (~2% false positive discoveries). In both tumor cohorts combined we observed a ~10-fold increase in CNA as compared to the controls (means 5042 and 519 oligonucleotide probes, respectively, $p \leq 0.0004$). The LTR and STR groups differed significantly (Fig. 1A, respective means 1864 and 7242, $p < 0.05$) and showed a statistically significant positive correlation between the groups and total CNAs (Fig. 1B, $p = 0.0014$; $r = 0.58$), implying that the adverse development of the disease may be linked to increase in chromosomal aberrations. A multivariate analysis was performed in order to distinguish whether CNA was an independent predictor of survival compared to other demographics including age, stage, sex, asbestos exposure, platelet count, and histology of the mesothelioma. Two models were considered: one in which stage was used as one of the variables and another in which stage was not. The second model was explored because in a number of cases where the mesothelioma would not have surgical therapy as in all of these cases, the stage would be unknown. We found that when stage was incorporated into the multivariate analysis it was by far the most important independent predictor of survival ($p = 0.0004$) with a 3.6 fold increase in relative risk of death for patients with Stages III/IV. In this model, platelets, histology, gender, age, histology, and a copy number abnormality greater than 5000 were not significant. However, in the model where stage is not incorporated, the CNA >5000 and

platelet count > 350 were independent predictors of survival ($p=0.01$ and $p=0.003$, respectively) with a relative risk of death 3.7 fold and 6.9 fold higher than for lower CNAs and platelet counts. Age, gender, histology, and lymph node status were not significant factors.

Validation of ROMA data

The capability of ROMA to detect copy number alterations in individual tumor specimens was validated using Affymetrix 250K NspI arrays. Five out of 22 DNA specimens used for ROMA were randomly chosen for a blinded CGH experiment. The ROMA and Affymetrix data were then compared sample-by-sample using moving average graphical output. The comparison showed a remarkable consistency in classification and mapping of the vast majority of the genomic imbalances, confirming the accuracy and reliability of ROMA (Suppl. Table 1). Among the five validated samples, several recurrent alterations were identified including losses in 1p and 9p (R342, R318, and R142), 3p (R342, R318), and 17pter (R318, R142, R342). All of these losses were classified by ROMA/ACE as highly frequent in our series of 22 cases (Table 2).

Mapping of most frequent chromosomal alterations and defining their minimal common regions

To increase sensitivity of the analysis the combined ROMA data for all 22 tumor specimens were plotted across human chromosomes 1-22 (Fig. 2). The resulting ACE profile was consistent with chromosomal instability (CIN) that affects most chromosomes. Some chromosomal regions, however, were more frequently involved than others as shown by prominent peaks in red (gains) and green (losses). Losses in chromosomes 22q12.2, 19, and 1p36 appeared to be the most frequent events (55-74%) in pleural MM followed by gains in 5p14-p15 and deletions in 17p and 9q (41-46%). Deletions in 1p13-21, 9p21, 3p14-21, and 17q21 as well as gains in 8q and 18q were observed in 27-36% of cases. Minimal common areas (MCAs) for these recurrent events were established through overlapping of individual deletions or gains (Fig. 3 and Table 2). Genes residing in the MCAs were identified using probe coordinates and Human Genome Database (NCBI Builds 36.2 and 35).

Cluster analysis of the STR and LTR group

The dramatic difference in the total number of genomic events observed between the STR and LTR groups prompted us to examine the relationship between and within these groups in more detail. Using ACE with an FDR of 0.023, we were able to isolate 85 probes, which discriminated significantly ($p<0.01$) between the STR and LTR groups and used them for cluster analysis (Fig. 4). The resulting dendrogram produced a clear distinction between the STR and LTR groups with only one patient, R166, misplaced. Inside the groups two samples, R158 and R258, did not cluster tightly within the respective group members. Notably, these two patients appear to be the only cases without a convincing history of asbestos exposure (Table 1). No discrimination between biphasic and epithelial types of MM was observed, and R125, the only sarcomatoid tumor in the study, clustered loosely with the rest of the group (Fig. 4).

Gene expression studies identify OSM, FUS1, PL6, CDH2, and DNAJA1 as differentially expressed during MM progression

Loss of chromosome 22, by far the most frequent event in our study (~74%, Table 2), was observed in both LTR and STR groups, suggesting that this is a common event in MM tumorigenesis. The MCA defined for 22q12.2 spans ~2 Mb and contains the oncostatin M gene (*OSM*), which encodes a proliferation-inhibiting cytokine. To find out if this gene's activity is related to MM progression, we performed RT-PCR analysis on additional tumor

and matched normal peritoneum specimens derived from 8 patients (Fig. 5A). *OSM* expression was seen in all normal samples and was not found in 5 tumor samples suggesting that it is, indeed, frequently suppressed in MM. Affymetrix expression array studies on an independent cohort of 30 MM and 7 normal peritoneum samples also confirmed our observation (Fig. 5B).

We also analyzed the 3p21.3 chromosomal area, which showed loss in 32% of cases. Our interest to this area was stimulated by its association with lung and other common cancers 17-18. We reported previously that loss in this area, although less precisely determined, can be observed in MM at ~40% frequency. In this study, we were able to discriminate between the 3p21.31 (~2.2 Mb) and 3p14.3-p14.2 (~1.1 Mb) areas and report that at least two candidate tumor suppressor genes from the 3p21.3 region, *FUS1(TUSC2)* and *PL6(TMEMI15)*, were ~2-fold down-regulated in 6 out of 8 tumornormal pleura matched specimens (Fig. 5A). Immunohistochemistry for the *FUS1* product using a novel antibody confirmed down-regulation of the gene (courtesy Ignatio Wistuba, MD Anderson Cancer Center, Houston, Texas, data not shown, manuscript in preparation).

Among the recurrent gains that we were able to identify in our series, the one in 18q12.1 contained the cadherin N gene (*CDH2*). Since cadherins play important roles in tumor progression, we studied *CDH2* expression using the same approach as for *OSM*. Up-regulation of *CDH2* was observed in a substantial fraction of additional sets of tumors (38%) by both RT-PCR and Affymetrix assays (Fig. 5AB) in agreement with the estimated frequency for this genomic gain (~36%, Table 2). 6 out of 8 ROMA specimens that showed the *CDH2* gain belonged to the STR group (Fig. 3B).

We also focused on *DNAJAI*, a member of the Hsp40 family of heat shock proteins. This gene is located in the smallest deletion detected in our study 9p21.1 microdeletion area (pos. 32780-33292 kb, Fig. 6A) that was established as the best discriminator between LTR and STR in a probe-by-probe statistical evaluation ($p < 0.007$). We have chosen *DNAJAI* as a cancer-related candidate since another gene from the same family, *DNAJB4*, was recently extensively studied in relation to non-small cell lung carcinoma and proved to be a tumor suppressor that shows LOH and transcriptional down-regulation in tumors 19. Using RNA samples isolated from tumor specimens used for ROMA we found that *DNAJAI* showed reduced expression in the STR cohort, which correlated with its allelic loss (4 out of 5 specimens with the deletions also showed down-regulation or lack of gene expression) pointing at the possible link between gene silencing and adverse clinical outcome (Fig. 6B). Affymetrix expression array analysis performed on the same set of tumor specimens (Fig. 6C) matched perfectly with the RT-PCR assessment.

DISCUSSION

Array CGH technologies provide new means for identification of most frequent (recurrent) DNA copy number imbalances in clinical specimens that contain a mix of normal and heterogeneous tumor genomes. In this study, we applied ROMA to clinical MM specimens to identify genetic events associated with disease progression. Cross-platform validation confirmed the reliability and accuracy of ROMA. To identify the most frequent aberrations in MM, we compiled and analyzed data generated on all 22 tumor specimens using ACE, a highly useful method of statistical processing of array-CGH data 20. This analysis showed multiple aberrations in the tumors from patients with fast relapse after surgery, estimated their frequencies, and accurately mapped them. The observed pattern suggests that adverse prognosis in MM is linked with a highly increased CIN. Fine mapping of the recurrent chromosomal events allowed us to identify genes localized within these areas, and associate them with the disease by expression analysis.

The critical MCAs shown in Table 2 are in agreement with previous karyotypic and LOH studies on MM (reviewed in 3). Loss of 9p21.3, observed by us in 32% of MM cases (Table 2), is one of the most thoroughly studied abnormalities in cancer and is linked to the tumor suppressors p16^{INK4a} and p14^{ARF} (encoded by alternative reading frames of *CDKN2A*), and p15^{INK4b} (encoded by *CDKN2B*) 21–23. About 85% of MM cell lines and more than 20% of MM tumor specimens show homozygous deletions in this chromosomal region 24. Mice deficient for all three 9p21.3 tumor suppressor genes are more tumor-prone and develop a wider spectrum of tumors than *Cdkn2a* mutant mice, with a preponderance of skin tumors and soft tissue sarcomas frequently composed of mixed cell types and often showing biphasic differentiation 21. In our study, the 9p21.3 deletion was observed exclusively in the STR cohort, substantiating the reported association with poor outcome 25. The genes were localized in the middle of the MCA for the region, underlining the high accuracy of our mapping (Fig. 2).

The reported high rate of chromosome 22 loss (~74%, Table 2) is consistent with chromosome 22 monosomy being the most frequent numerical change in MM 3. Allelic loss and mutations in *NF2*, located at 22q12.2, have been observed in ~50% of MMs 27. Our MCA established for chromosome 22 points at a neighboring gene, *OSM*, in the same area (Table 2). *OSM* encodes a cytokine known as oncostatin M, which possesses growth suppressor propensity. Notably, *OSM* expression was silenced in 5 out of 8 paired tumor/normal specimens derived from MM patients (Fig. 5A). Growth suppressive effect of *OSM* was documented for melanoma, glioblastoma, breast, lung, and prostatic cancer cell lines 26–30 but has not been previously implicated in MM. We hypothesize that co-deletion of *OSM* with *NF2* may be advantageous for tumor progression.

Loss of chromosome 17p is also a common and important event in MM progression (our estimation of 46% is similar to the 40% reported in the literature 3) since the p53 gene *TP53* is localized in 17p13.1. The MCA generated for the 17p (4791–5367 Kb, Table 2) by ACE, however, does not include *TP53*, suggesting the existence of a yet unknown tumor suppressor gene.

The 3p21.3 deletion is involved in many malignancies including asbestos-associated lung cancers 31 suggesting its significance for common tumorigenesis 17–18. The region is densely packed with genes and may contain several potential tumor suppressors 32. One of the genes located in the area, *RASSF1A*, has been already established to encode a pro-apoptotic tumor suppressor 33 and its loss is associated with many cancers including MM 34. According to the most recent studies, at least two more genes from the same area, *FUS1* (*TUSC2*) and *PL6* (*TMEM115*), may be also associated with cancer. Forced expression of *FUS1* resulted in tumor growth suppression 35, and its disruption in a mouse model produced a compromised immune response and increased susceptibility to spontaneous cancers 36. Association of *FUS1* expression with stimulation of p53-regulated apoptosis provided an important insight into its tumor suppressor activity 37. Silencing of *PL6* observed in all clinical specimens of renal clear cell carcinoma (n=57) and other VHL-dependent malignancies suggests its involvement in early stages of VHL-deficient cancers 38. Here we show that both *FUS1* and *PL6* are consistently down regulated in MM at the mRNA level (Fig. 5A), in agreement with their roles as tumor suppressors.

We also identified three chromosomal areas that, to our knowledge, have never been reported in connection with MM. One of these events, loss of chromosome 19, was the second most frequent numerical change after chromosome 22 (55 versus 74%, Table 2) implying its critical importance for MM. Remarkably, deletions that coincide with our MCA area (19p13) were recently found in asbestos-induced lung cancer and associated with the genotoxic effect of asbestos 31. The region we defined contains at least 15 potential cancer-

associated genes (Table 2). Further research is needed to provide clues as to which of them are most likely asbestos exposure-associated tumor suppressors.

Loss in chromosome 9q34.11 (41%, Table 2) is yet another novel finding that allowed us to consider 3 of 9 genes identified in the MCA area, *STXBPI*, *SH2D3C* and *ENG*, as plausible tumor suppressor candidates. Experiments performed on a mouse skin cancer model suggested that *ENG*, which encodes the TGF- β coreceptor endoglin, may indeed act as a tumor suppressor during the late stages of carcinogenesis 39. Among the recurrent gains that we identified, those in chromosomes 5p, 8q, and 17q have been already characterized 3⁴⁰, while the remaining one in 18q12.1 appeared to be novel. Assessment of the 18q12.1 gain that we found predominantly in the STR patients (6 STR versus 2 LTR) identified one gene (*CDH2*) in the area. *CDH2* encodes N-cadherin, which was upregulated in MM samples (Fig. 5A). Expression of N-cadherin in MM was reported previously at the protein level and used for discrimination of MM from lung adenocarcinoma 41⁴⁴. Overexpression of adhesion-associated N-cadherin was also observed in hepatocellular carcinoma 45, renal cancer 46, invasive retinoblastoma 47, and melanoma 48⁴⁹. In the latter case, N-cadherin expression was casually linked with transendothelial migration of melanoma cells and associated with Notch1 signaling. Studies on pancreatic cancer, one of the most aggressive malignancies, suggest that N-cadherin plays a principal role in epithelial-to-mesenchymal transition, which, in turn, is linked with invasion and metastasis 50. A peptide that blocks N-cadherin prevented tumor growth and spread in a mouse skin cancer model 51. Overexpression of *CDH2* in MM and a high correlation observed between expression of *CDH2* and *MKI57*, a proliferation-specific marker that encodes Ki-67 52 (Fig. 5A), is consistent with the gain observed in 18q12.1 and suggests an oncogenic role for N-cadherin in MM.

In conclusion, by combining a highly sensitive ROMA assay with novel noise-reducing statistical software, we have been able to precisely profile DNA copy number changes typical of MM and have identified recurrent genomic imbalances implicating genes that may be associated with early stages of the disease and its adverse prognosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported in part by funds from the Early Detection Research Network NCI, NIH to Dr. Pass' Mesothelioma Biomarker Discovery Laboratory, by NCI grants CA114047 and CA06927, by a grant from the Mesothelioma Applied Research Foundation, Santa Barbara, California, by NIH grant CA06927 and the Commonwealth of Pennsylvania, and by philanthropy from Belluck and Fox, LLP New York, New York.

Abbreviations

MM	malignant pleural mesothelioma
CNA	copy number alterations
MCA	minimal common area
CIN	chromosomal instability

REFERENCES

1. Marinaccio A, Branchi C, Massari S, Scarselli A. National epidemiologic surveillance systems of asbestos-related disease and the exposed workers register. *Med Lav.* 2006; 97:482–7. [PubMed: 17009684]
2. Pass HI, Kranda K, Temeck BK, Feuerstein I, Steinberg SM. Surgically debulked malignant pleural mesothelioma: results and prognostic factors. *Ann Surg Oncol.* 1997; 4:215–22. [PubMed: 9142382]
3. Apostolou SBBR, Testa JR. Cytogenetics of Malignant Mesotheliomaed. 2005
4. Pinkel D, Albertson DG. Array comparative genomic hybridization and its applications in cancer. *Nat Genet.* 2005; 37(Suppl):S11–7. [PubMed: 15920524]
5. Gebhart E. Comparative genomic hybridization (CGH): ten years of substantial progress in human solid tumor molecular cytogenetics. *Cytogenet Genome Res.* 2004; 104:352–8. [PubMed: 15162064]
6. Balsara BR, Bell DW, Sonoda G, De Rienzo A, du Manoir S, Jhanwar SC, Testa JR. Comparative genomic hybridization and loss of heterozygosity analyses identify a common region of deletion at 15q11.1-15 in human malignant mesothelioma. *Cancer Res.* 1999; 59:450–4. [PubMed: 9927061]
7. De Rienzo A, Testa JR. Recent advances in the molecular analysis of human malignant mesothelioma. *Clin Ter.* 2000; 151:433–8. [PubMed: 11211478]
8. Davies JJ, Wilson IM, Lam WL. Array CGH technologies and their applications to cancer genomes. *Chromosome Res.* 2005; 13:237–48. [PubMed: 15868418]
9. Lucito R, West J, Reiner A, Alexander J, Esposito D, Mishra B, Powers S, Norton L, Wigler M. Detecting gene copy number fluctuations in tumor cells by microarray analysis of genomic representations. *Genome Res.* 2000; 10:1726–36. [PubMed: 11076858]
10. Lucito R, Healy J, Alexander J, Reiner A, Esposito D, Chi M, Rodgers L, Brady A, Sebat J, Troge J, West JA, Rostan S, et al. Representational oligonucleotide microarray analysis: a high-resolution method to detect genome copy number variation. *Genome Res.* 2003; 13:2291–305. [PubMed: 12975311]
11. Lucito, R.; Wigler, M. Preparation of target DNAed. Cold Spring Harbor Press; 2003b.
12. Lucito, R.; Wigler, M. Preparation of slides and hybridizationed. Cold Spring Harbor Press; 2003a.
13. Lingjaerde OC, Baumbusch LO, Liestol K, Glad IK, Borresen-Dale AL. CGH-Explorer: a program for analysis of array-CGH data. *Bioinformatics.* 2005; 21:821–2. [PubMed: 15531610]
14. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. *Behav Brain Res.* 2001; 125:279–84. [PubMed: 11682119]
15. Gordon GJ, Jensen RV, Hsiao LL, Gullans SR, Blumenstock JE, Richards WG, Jaklitsch MT, Sugarbaker DJ, Bueno R. Using gene expression ratios to predict outcome among patients with mesothelioma. *J Natl Cancer Inst.* 2003; 95:598–605. [PubMed: 12697852]
16. Ivanov SV, Salnikow K, Ivanova AV, Bai L, Lerman MI. Hypoxic repression of STAT1 and its downstream genes by a pVHL/HIF-1 target DEC1/STRA13. *Oncogene.* 2007; 26:802–12. [PubMed: 16878149]
17. Lerman MI, Minna JD. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. *Cancer Res.* 2000; 60:6116–33. [PubMed: 11085536]
18. Hesson LB, Cooper WN, Latif F. Evaluation of the 3p21.3 tumour-suppressor gene cluster. *Oncogene.* 2007
19. Tsai MF, Wang CC, Chang GC, Chen CY, Chen HY, Cheng CL, Yang YP, Wu CY, Shih FY, Liu CC, Lin HP, Jou YS, et al. A new tumor suppressor DnaJ-like heat shock protein, HLJ1, and survival of patients with non-small-cell lung carcinoma. *J Natl Cancer Inst.* 2006; 98:825–38. [PubMed: 16788156]
20. Lai WR, Johnson MD, Kucherlapati R, Park PJ. Comparative analysis of algorithms for identifying amplifications and deletions in array CGH data. *Bioinformatics (Oxford, England).* 2005; 21:3763–70.

21. Krimpenfort P, Ijpenberg A, Song JY, van der Valk M, Nawijn M, Zevenhoven J, Berns A. p15Ink4b is a critical tumour suppressor in the absence of p16Ink4a. *Nature*. 2007; 448:943–6. [PubMed: 17713536]
22. Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA. Role of the INK4a locus in tumor suppression and cell mortality. *Cell*. 1996; 85:27–37. [PubMed: 8620534]
23. Frizelle SP, Grim J, Zhou J, Gupta P, Curiel DT, Geradts J, Kratzke RA. Re-expression of p16INK4a in mesothelioma cells results in cell cycle arrest, cell death, tumor suppression and tumor regression. *Oncogene*. 1998; 16:3087–95. [PubMed: 9671387]
24. Cheng JQ, Jhanwar SC, Klein WM, Bell DW, Lee WC, Altomare DA, Nobori T, Olopade OI, Buckler AJ, Testa JR. p16 alterations and deletion mapping of 9p21-p22 in malignant mesothelioma. *Cancer Res*. 1994; 54:5547–51. [PubMed: 7923195]
25. Lopez-Rios F, Chuai S, Flores R, Shimizu S, Ohno T, Wakahara K, Illei PB, Hussain S, Krug L, Zakowski MF, Rusch V, Olshen AB, et al. Global gene expression profiling of pleural mesotheliomas: overexpression of aurora kinases and P16/CDKN2A deletion as prognostic factors and critical evaluation of microarray-based prognostic prediction. *Cancer Res*. 2006; 66:2970–9. [PubMed: 16540645]
26. Horn D, Fitzpatrick WC, Gompfer PT, Ochs V, Bolton-Hansen M, Zarling J, Malik N, Todaro GJ, Linsley PS. Regulation of cell growth by recombinant oncostatin M. *Growth Factors*. 1990; 2:157–65. [PubMed: 2160258]
27. Zhang F, Li C, Halfter H, Liu J. Delineating an oncostatin M-activated STAT3 signaling pathway that coordinates the expression of genes involved in cell cycle regulation and extracellular matrix deposition of MCF-7 cells. *Oncogene*. 2003; 22:894–905. [PubMed: 12584569]
28. Underhill-Day N, Heath JK. Oncostatin M (OSM) cytostasis of breast tumor cells: characterization of an OSM receptor beta-specific kernel. *Cancer Res*. 2006; 66:10891–901. [PubMed: 17108126]
29. Lu C, Rak JW, Kobayashi H, Kerbel RS. Increased resistance to oncostatin M-induced growth inhibition of human melanoma cell lines derived from advanced-stage lesions. *Cancer Res*. 1993; 53:2708–11. [PubMed: 8504408]
30. Friedrich M, Hoss N, Stogbauer F, Senner V, Paulus W, Ringelstein EB, Halfter H. Complete inhibition of in vivo glioma growth by oncostatin M. *J Neurochem*. 2001; 76:1589–92. [PubMed: 11238743]
31. Wikman H, Ruosaari S, Nymark P, Sarhadi VK, Saharinen J, Vanhala E, Karjalainen A, Hollmen J, Knuutila S, Anttila S. Gene expression and copy number profiling suggests the importance of allelic imbalance in 19p in asbestos-associated lung cancer. *Oncogene*. 2007; 26:4730–7. [PubMed: 17297452]
32. Ji L, Minna JD, Roth JA. 3p21.3 tumor suppressor cluster: prospects for translational applications. *Future Oncol*. 2005; 1:79–92. [PubMed: 16555978]
33. Matallanas D, Romano D, Yee K, Meissl K, Kucerova L, Piazzolla D, Baccarini M, Vass JK, Kolch W, O'Neill E. RASSF1A elicits apoptosis through an MST2 pathway directing proapoptotic transcription by the p73 tumor suppressor protein. *Mol Cell*. 2007; 27:962–75. [PubMed: 17889669]
34. Toyooka S, Carbone M, Toyooka KO, Bocchetta M, Shivapurkar N, Minna JD, Gazdar AF. Progressive aberrant methylation of the RASSF1A gene in simian virus 40 infected human mesothelial cells. *Oncogene*. 2002; 21:4340–4. [PubMed: 12082623]
35. Ji L, Nishizaki M, Gao B, Burbee D, Kondo M, Kamibayashi C, Xu K, Yen N, Atkinson EN, Fang B, Lerman MI, Roth JA, et al. Expression of several genes in the human chromosome 3p21.3 homozygous deletion region by an adenovirus vector results in tumor suppressor activities in vitro and in vivo. *Cancer Res*. 2002; 62:2715–20. [PubMed: 11980673]
36. Ivanova AV, Ivanov SV, Pascal V, Lumsden JM, Ward JM, Morris N, Tessarolo L, Anderson SK, Lerman MI. Autoimmunity, spontaneous tumorigenesis, and IL-15 insufficiency in mice with a targeted disruption of the tumour suppressor gene *Fus1*. *J Pathol*. 2007; 211:591–601. [PubMed: 17318811]
37. Deng WG, Kawashima H, Wu G, Jayachandran G, Xu K, Minna JD, Roth JA, Ji L. Synergistic tumor suppression by coexpression of *FUS1* and *p53* is associated with down-regulation of murine double minute-2 and activation of the apoptotic protease-activating factor 1-dependent apoptotic

- pathway in human non-small cell lung cancer cells. *Cancer Res.* 2007; 67:709–17. [PubMed: 17234782]
38. Ivanova A, Vortmeyer A, Ivanov S, Nickerson M, Maher E, Lerman M. Loss of PL6 protein expression in renal clear cell carcinomas and other VHL-deficient tumours. *J Pathol.* 2007
 39. Perez-Gomez E, Villa-Morales M, Santos J, Fernandez-Piqueras J, Gamallo C, Dotor J, Bernabeu C, Quintanilla M. A role for endoglin as a suppressor of malignancy during mouse skin carcinogenesis. *Cancer Res.* 2007; 67:10268–77. [PubMed: 17974968]
 40. Kivipensas P, Bjorkqvist AM, Karhu R, Pelin K, Linnainmaa K, Tammilehto L, Mattson K, Kallioniemi QP, Knuutila S. Gains and losses of DNA sequences in malignant mesothelioma by comparative genomic hybridization. *Cancer Genet Cytogenet.* 1996; 89:7–13. [PubMed: 8689616]
 41. Han AC, Filstein MR, Hunt JV, Soler AP, Knudsen KA, Salazar H. N-cadherin distinguishes pleural mesotheliomas from lung adenocarcinomas: a ThinPrep immunocytochemical study. *Cancer.* 1999; 87:83–6. [PubMed: 10227598]
 42. Han AC, Peralta-Soler A, Knudsen KA, Wheelock MJ, Johnson KR, Salazar H. Differential expression of N-cadherin in pleural mesotheliomas and E-cadherin in lung adenocarcinomas in formalin-fixed, paraffin-embedded tissues. *Hum Pathol.* 1997; 28:641–5. [PubMed: 9190996]
 43. Pelin K, Hirvonen A, Linnainmaa K. Expression of cell adhesion molecules and connexins in gap junctional intercellular communication deficient human mesothelioma tumour cell lines and communication competent primary mesothelial cells. *Carcinogenesis.* 1994; 15:2673–5. [PubMed: 7955125]
 44. Soler, A Peralta; Knudsen, KA.; Jaurand, MC.; Johnson, KR.; Wheelock, MJ.; Klein-Szanto, AJ.; Salazar, H. The differential expression of N-cadherin and E-cadherin distinguishes pleural mesotheliomas from lung adenocarcinomas. *Hum Pathol.* 1995; 26:1363–9. [PubMed: 8522310]
 45. Jiang C, Pecha J, Hoshino I, Ankrapp D, Xiao H. TIP30 mutant derived from hepatocellular carcinoma specimens promotes growth of HepG2 cells through up-regulation of N-cadherin. *Cancer Res.* 2007; 67:3574–82. [PubMed: 17440068]
 46. Shimazui T, Kojima T, Onozawa M, Suzuki M, Asano T, Akaza H. Expression profile of N-cadherin differs from other classical cadherins as a prognostic marker in renal cell carcinoma. *Oncol Rep.* 2006; 15:1181–4. [PubMed: 16596183]
 47. Mohan A, Nalini V, Mallikarjuna K, Jyotirmay B, Krishnakumar S. Expression of motility-related protein MRP1/CD9, N-cadherin, E-cadherin, alpha-catenin and beta-catenin in retinoblastoma. *Exp Eye Res.* 2007; 84:781–9. [PubMed: 17316610]
 48. Qi J, Chen N, Wang J, Siu CH. Transendothelial migration of melanoma cells involves N-cadherin-mediated adhesion and activation of the beta-catenin signaling pathway. *Mol Biol Cell.* 2005; 16:4386–97. [PubMed: 15987741]
 49. Liu ZJ, Xiao M, Balint K, Smalley KS, Brafford P, Qiu R, Pinnix CC, Li X, Herlyn M. Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. *Cancer Res.* 2006; 66:4182–90. [PubMed: 16618740]
 50. Nakajima S, Doi R, Toyoda E, Tsuji S, Wada M, Koizumi M, Tulachan SS, Ito D, Kami K, Mori T, Kawaguchi Y, Fujimoto K, et al. N-cadherin expression and epithelial-mesenchymal transition in pancreatic carcinoma. *Clin Cancer Res.* 2004; 10:4125–33. [PubMed: 15217949]
 51. Shintani Y, Fukumoto Y, Chaika N, Grandgenett PM, Hollingsworth MA, Wheelock MJ, Johnson KR. ADH-1 suppresses N-cadherin-dependent pancreatic cancer progression. *Int J Cancer.* 2008; 122:71–7. [PubMed: 17721921]
 52. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *Journal of cellular physiology.* 2000; 182:311–22. [PubMed: 10653597]

Fig. 1. Accumulation of CNAs in MM

Differentiation of three groups of specimens by total CNA number (A) and by correlation analysis (B).

Fig. 2. Chromosomal localization and frequencies of imbalances in 22 MM tumors as assessed with ACE (FDR=0.023)

Losses in tumor versus normal control are shown in green and gains are in red. Sex chromosomes are omitted from the study.

Fig. 3. Fine mapping of losses in 9p21.3 (A) and gains in 18q12.1 (B) regions

A, From top to bottom: STR specimens R140, R249, R367, R312, R318, R342, and R142 show overlapping deletions (in green). Minimal common area (MCA) and genes located therein are shown. **B**, STR specimens R249, R367, R312, R318, R342 and R142, and LTR specimens R219 and R298 show overlapping gains (in red). LTR sample R140 shows loss in the same region, depicted in green.

Fig. 4.
Hierarchical clustering of MM specimens based on 85 probes that distinguish between STR and LTR

Fig. 5. Validation of gene candidates by RT-PCR (A) and Affymetrix expression analysis (B)
A, Matched normal (N) and tumor (T) specimens derived from eight MM patients were used for RT-PCR. *MKI67*, encoding Ki-67, was used as a proliferation marker and invariantly expressed *PPIA* as a loading control. **B**, Expression array validation on an additional set of MM (n=30) and normal pleura (n=7) specimens for on the U133 2.0 Plus platform.

Fig. 6. Association of *DNAJA1* with STR MM by array-CGH (A), RT-PCR on ROMA specimens (B), and Affymetrix expression study (C) *DNAJA1* location in the 9p21.3 MCA is shown. Deletions in this area are shown in green, and the respective samples on the gel are marked with “d”.

Table1

Demographics of MM patients and their CNA estimations (FDR=0.023).

Specimen	Group	Gender	Age	Histology	Stage	Asbestos	Time to recurrence (death)	Total CNA
R158	LTR	F	81	biphasic	1	N	15(24)	577
R219	LTR	M	49	biphasic	1	Y	68	920
R298	LTR	M	79	epithelial	1	Y	22.5(48)	6183
R322	LTR	F	76	epithelial	1	Y	30 (63)	1541
R374	LTR	M	55	epithelial	1	Y	16(55)	1876
R144	LTR	M	77	biphasic	3	Y	14(20)	453
R125	LTR	M	78	sarcomatoid	2	Y	81	4702
R143	LTR	M	55	epithelial	2	Y	77	285
R351	LTR	M	78	biphasic	2	Y	20(41)	237
R166	STR	M	71	biphasic	2	Y	6(25)	1161
R312	STR	M	74	biphasic	2	Y	12(15)	4545
R140	STR	M	58	epithelial	3	Y	5(11)	5672
R142	STR	M	53	epithelial	3	Y	3(9)	17409
R172	STR	M	62	epithelial	3	Y	2(23)	9626
R184	STR	M	65	epithelial	3	Y	12(41)	9912
R194	STR	M	74	epithelial	3	Y	9(12)	2344
R249	STR	M	63	epithelial	3	Y	12(14)	7538
R258	STR	M	68	epithelial	3	N	7(27)	1255
R336	STR	M	49	epithelial	3	Y	4(5)	1094
R342	STR	M	48	epithelial	3	Y	5(9)	11670
R367	STR	M	63	biphasic	3	Y	4(3)	6102
R318	STR	M	62	biphasic	2	Y	11(37)	15822
R125N	NORM	M						740

Specimen	Group	Gender	Age	Histology	Stage	Asbestos	Time to recurrence (death)	Total CNA
R143N	NORM	M						479
R312N	NORM	M						725
R336N	NORM	M						482
R291N	NORM	M						167

Table 2
Major losses and gains detected in MM

Genes validated by expression studies are shown in bold and underlined.

Chromosomal region	Gain or loss	Position in kb	Frequency , %	Putative tumor suppressors or oncogenes
1p36.22-p36.23	loss	7816-11381	36	TNF RSF9, ERFF1(MIG6), GPR157, PIK3CD, TNFRSF25, CTNNBIP1, APITD1, DFFA, CASZ1
1p36.11-p36.12	loss	22168-24471	55	CDC42, WNT4, ZBTB40, EPHA8, EPHB2, LUZP1, DDEFL1
1p13.2-p13.3	loss	109760-112212	36	GPR61, GNAI3, EPS8L3, RAP1A
3p21.31	loss	49500-51691	27	IHPK1, SEMA3F, GNAI2, SEMA3B, IRFD2, FUS1 , RASSF1, ZMYND10, TUSC4, PL6 CISH, MAPKAPK3, DOCK3
3p14.3-p14.2	loss	56682-57839	32	ARHGEF3, IL17RD, HESX1, APPL1, ASB14, ARF4,
9p21.3	loss	19013-24303	32	RRAGA, PTPLAD2, CDKN2A, CDKN2B, DMRTA1, PLAA, MOBKL2B
9p21.1	loss	32828-33021	36	APTX, DNAJA1
9q34.11	loss	129384-129681	41	METTL11A, ASB6, PRRX2, PTGES, DENND1C
17p13.1	loss	4791-5367	46	ARRB2, MINK1, PFN1, INCA1, GPR172B, RABEP1, USP6, MIS12
17q21.31	loss	41074-41462	32	HDAC5, TMUB2, SHCL1, RPIP8, FZD2, DBF4B, HIGDB1, IMP5, MAPT, STH, CDC27
19p13.2	loss	4887-9263	55	PTPRS, SAFB2, SAFB, RFX2, MLLT1, CRB3, TNFSF9, TNFS7, TNFS14, ARHGEF18, MAP2K7
19q13.32	loss	49881-55635	55	MARK4, ERCC2, PPP1R13L, ERCC1, GPR4, EML2, DMPK, IRF2BP1, PPP5C, PRKD2, GNG8, DACT3, BBC3, GPR77, MEIS3, GLTSCR1, GLTSR2, CARD8, PPFA3, LMTK3, SPHK2, RASIP1, BAX, LIN7B, PPP1R15A, DKKL1, IRF3, BCL2L12, PNKP
22q12.2	loss	28729-29466	74	HORMAD2, LIF, OSM , TBC1D10A, PTPNS1L, DUSP18
5p14	gain	25445-26754	55	MSNL1
17q21-q23	gain	46947-52081	24	TOM1L1, HLF, ANKFN1
8q23-q24	gain	112442-113551	36	CSMD3
18q12.1	gain	23870-25900	36	CDH2 , LOC390844, LOC440490