Cellular Oncology 27 (2005) 169–173
IOS Press

Review

NMD microarray analysis for rapid genome-wide screen of mutated genes in cancer

Maija Wolf [a,*], Henrik Edgren [a], Aslaug Muggerud [b], Sami Kilpinen [a], Pia Huusko [c], Therese Sørlie [b], Spyro Mousses [c] and Olli Kallioniemi [a]

[a] *Medical Biotechnology, VTT Technical Research Centre of Finland and University of Turku, FIN-20520 Turku, Finland*
[b] *Department of Genetics, The Norwegian Radium Hospital, N-0310 Oslo, Norway*
[c] *Translational Genomics Research Institute, Gaithersburg, MD 20878-1762, USA*

Abstract. Gene mutations play a critical role in cancer development and progression, and their identification offers possibilities for accurate diagnostics and therapeutic targeting. Finding genes undergoing mutations is challenging and slow, even in the post-genomic era. A new approach was recently developed by Noensie and Dietz to prioritize and focus the search, making use of nonsense-mediated mRNA decay (NMD) inhibition and microarray analysis (NMD microarrays) in the identification of transcripts containing nonsense mutations. We combined NMD microarrays with array-based CGH (comparative genomic hybridization) in order to identify inactivation of tumor suppressor genes in cancer. Such a "mutatomics" screening of prostate cancer cell lines led to the identification of inactivating mutations in the *EPHB2* gene. Up to 8% of metastatic uncultured prostate cancers also showed mutations of this gene whose loss of function may confer loss of tissue architecture. NMD microarray analysis could turn out to be a powerful research method to identify novel mutated genes in cancer cell lines, providing targets that could then be further investigated for their clinical relevance and therapeutic potential.

Keywords: NMD, microarray, mutation, tumor suppressor gene

1. Mutations and cancer

Mutations leading to the deregulation of critical genes are thought to underlie the multi-step process contributing to cancer development [33]. These may involve activating mutations in proto-oncogenes (e.g. *EGFR, BRAF*), and/or inactivating mutations in tumor suppressor (e.g. *RB1, APC*) and "caretaker genes" (e.g. *BRCA1, MSH2*). DNA sequence analysis of such genes offers both opportunities for diagnosing cancer predisposition, for somatic cancer diagnosis, and for targeting therapeutics towards tumors with specific, clonally selected genetic alterations. Only roughly 1% of genes are known to harbor mutations in cancer [8] and only a handful of these are relevant to the diagnosis or therapeutic management of common solid tumors, such as the common epithelial cancers. Finding new mutated genes is slow and labor-intensive, even in the genomic era. For example, there are dozens if not hundreds of specific sites for deletions and allelic losses along chromosomal arms, but very few of these have yet been associated with mutated tumor suppressor genes. Therefore, more effective methods to identify genes with DNA sequence alterations are needed. We, among several other groups, have recently illustrated the potential of the nonsense-mediated mRNA decay (NMD) microarray strategy in highlighting putative mutated genes in cancer [11,13,29].

2. Nonsense-mediated mRNA decay (NMD)

The NMD-pathway is evolutionarily well-conserved and functions to protect cells from the deleterious ef-

*Corresponding author: Maija Wolf, Medical Biotechnology, VTT Technical Research Centre of Finland and University of Turku, Itäinen Pitkäkatu 4, FIN-20520 Turku, Finland. Tel.: +358 9 471 71916; Fax: +358 9 471 71731; E-mail: maija.wolf@vtt.fi.

fects of mutations and errors in the transcription leading to premature termination codons (PTCs) [4,21]. Accumulation of truncated proteins could act in a dominant-negative manner or otherwise interfere with normal cellular functions. It has been estimated that about one third of the mutations associated with human diseases are nonsense mutations that result in PTCs [10,23].

In eukaryotes, messenger RNAs containing nonsense mutations are selectively and rapidly degraded by the NMD-pathway during translation [21,24]. In mammalian cells, NMD is linked to splicing and involves the formation of exon-junction-complex (EJC) 20–24 nucleotides 5′ of each exon–exon junction [2,17]. EJC acts together with a group of Upf proteins to trigger the formation of a multi-protein surveillance complex after splicing [22]. The complexes are normally displaced from the mRNA by the first translating ribosome. In case of a PTC, at least one of the surveillance complexes remains bound to the mRNA, leading to phosphorylation of UPF1/RENT1 and activation of the NMD machinery [5,35], details of which remain partly unknown. In order to elicit NMD, the PTC must lie more than 50 to 55 nucleotides upstream of the last exon–exon junction [3,28]. In addition, close proximity of PTC to the translation initiation codon has been postulated to interfere with the activation of NMD [12]. Activation of NMD results in rapid transcript degradation through deadenylation of the poly(A) tail followed by 3′-5′ degradation, and/or decapping of the mRNA followed by 5′-3′ degradation [18,31]. Most PTC containing mRNAs are subject to nucleus-associated NMD, whereas minority of NMD driven mRNA degradation occurs in the cytoplasm [25].

Recent views suggest that instead of acting merely as a quality control mechanism, NMD may function primarily as a regulator of physiological transcripts. Such physiologic substrates for NMD have been shown to include transcripts with alternative splice forms that introduce nonsense codons or frameshift mutations to the spliced sequence, open reading frames in the 5′ untranslated region and introns in the 3′ untranslated region [7,20,26,30,34]. NMD regulated transcripts have been shown to include particularly those involved in amino acid metabolism, immune maturation, telomere capping, embryonic development, as well as those encoding selenoproteins [19,26,27].

3. Manipulation of the NMD pathway for mutation screening

Noensie and Dietz initially proposed the "GINI" (Gene Identification by NMD Inhibition) technology for the discovery of mutations without any a priori information on the disease or the genes of interest [29]. In this method, the NMD pathway is blocked pharmacologically by treating the cells with a translational inhibitor, such as emetine, or by siRNAs against Rent1 [26,29], a key protein involved in the NMD process. As a consequence of such treatment, mutated transcripts containing PTCs are stabilized and accumulate in the cells during culture. The corresponding enrichment in mRNA abundance can be monitored by comparing mRNA levels before and after treatment using standard gene expression microarrays. This makes it possible to identify transcripts that are stabilized by the NMD inhibition.

The use of translation inhibitors, such as emetine, prolongs the half-lives of numerous mRNAs, not just those having PTCs, introducing "noise" to the data [32]. Bioinformatic analysis of the inhibition data, along with data from untreated cells, should therefore be used to facilitate distinction of transcripts with truncating mutations from physiologic substrates for NMD ("genomic noise") as well as transcripts that are otherwise stabilized or up-regulated by the drug or siRNA treatment. Several methods can be used to filter out nonPTC containing emetine induced transcripts. One approach is to assume any given gene is mutated in only a fraction of samples. Genes induced in more than one or two cell lines can then be excluded as likely physiological NMD substrates or as genes that are transcriptionally induced by emetine. Normal cell lines would be especially informative, since they are not expected to contain PTC mutations. In practice, comparison of our unpublished results to those of Ionov and colleagues [13] has shown that a significant fraction of emetine induced genes are the same in different cell lines. Emetine induced genes can also be excluded if they contain specific sequence features, such as 5′ open reading frames or 3′ introns, which are found in physiological NMD substrates.

In the context of a large international consortium, we recently applied the NMD technique to screen for mutated transcripts in prostate cancer cell lines [11]. Studies of chromosomal deletions in tumor tissues and cell lines have identified dozens of specific genomic regions that may harbor yet to be identified tumor-suppressor genes important for prostate cancer progression [6]. These altered regions contain, however, hundreds of candidate genes making the positional discovery of disease-causing mutations a challenging effort. As bi-allelic inactivation of tumor suppressor genes often involves deletion of the wild-type

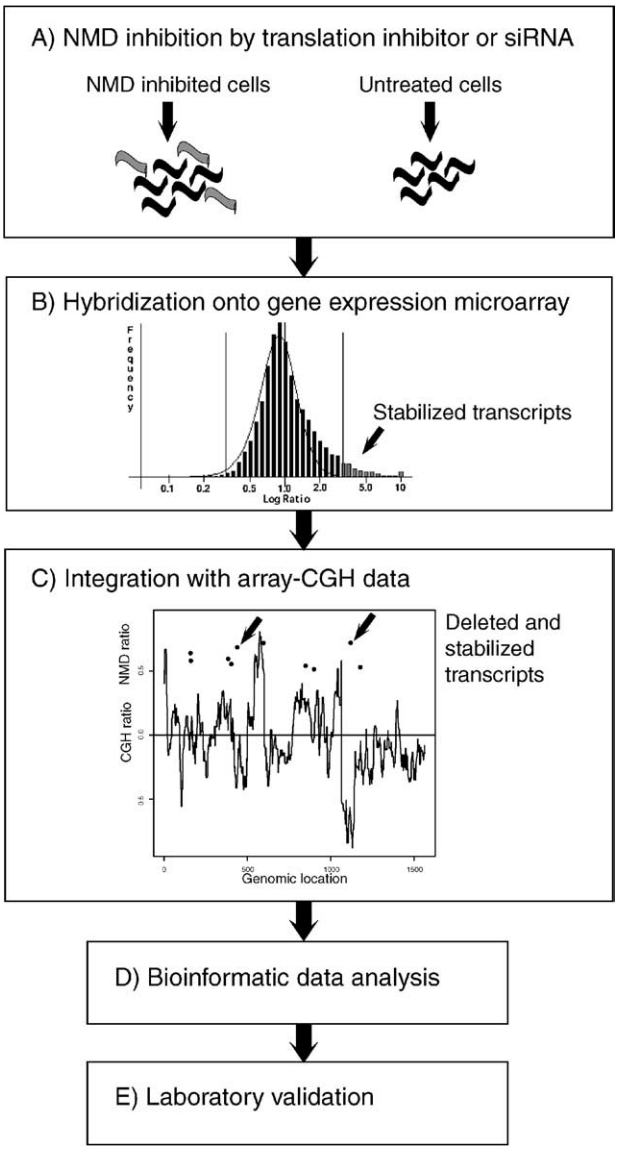

Fig. 1. Schematic workflow for NMD microarray-based screening: (A) NMD pathway can be blocked by using translation inhibitors or siRNAs against key NMD pathway components. Reference cells are left untreated. (B) After RNA isolation, reverse transcription and labeling of the two RNA populations with different fluorescent dyes, gene expression microarray analysis is carried out and stabilized transcripts are identified. (C) CGH microarrays are used to determine deleted loci from the same sample. Integration with NMD inhibition data allows pinpointing both stabilized and deleted genes from the data set. (D) Lists of candidate genes are prioritized using bioinformatic data analysis. This includes filtering out physiologic NMD substrates and common stabilized genes due to drug or siRNA treatment unrelated to mutation status. (E) Truncating mutations or alternative splicing of the selected genes are verified from the studied samples, and the clinical relevance of the detected alterations are determined from patient samples.

allele in addition to mutation of the other allele [15], we integrated the NMD microarray data with comparative genomic hybridization (CGH) microarray data. This makes it possible to focus the search of putative mutated tumor suppressor genes to the deleted chromosomal regions of the cancer cells as visualized by high-resolution CGH microarray data. Figure 1 shows a schematic overview of the NMD microarray-based analysis for mutation detection.

Application of this integrated NMD-CGH method led to the ∼1000-fold enrichment of known TSG mutations in the analyzed cancer cell lines, and to the discovery of a new tumor suppressor candidate, *EPHB2*, which is mutated in 8% of patient samples from ad-

vanced and metastatic prostate cancers. Furthermore, reconstitution of the wild-type EPHB2 in the DU 145 prostate cancer cells suppressed clonogenic growth, providing functional evidence of the tumor suppressing properties of this gene [11]. EPHB2 belongs to the largest family of receptor tyrosine kinases that are involved in cell-to-cell communication. EPHB2 has been postulated to control positioning of proliferative and differentiated cells in normal tissues, such as in the colorectal crypts [1,9,16]. *EPHB2* may therefore be a critical gene whose loss explains the dysregulation of tissue architecture, one of the hallmarks of cancer.

Cancer cell lines manifesting microsatellite instability (MSI) are prone to accumulate mutations, especially frameshifts leading to downstream stop codons at numerous sites in the genome. Ionov and collaborators reported the application of the NMD microarray analysis in colorectal cancer cell lines with MSI [13,14]. In addition to emetine, cells were treated simultaneously with actinomycin D, a transcription inhibitor, to prevent new transcription and, as a result, minimizing the effect of emetine-based stress response [13]. Using this strategy, candidate mutated genes were identified. Sequence alterations were verified in genes encoding UVRAG and p300, the first having a putative role in DNA repair process and the latter, functioning as a histone acyltransferase, with a regulatory effect on p53 activity [14].

4. Future prospects

Manipulating the NMD-pathway, followed by microarray analyses has already been applied in various discovery approaches for mutated genes. The first published studies [11,13,26,29] illustrate the potential of this approach as a research tool. Typically, each NMD analysis creates a long list of candidate genes to study, including many false positive hits. Several hits may reflect alternatively spliced gene products, that may be informative on their own, rather than nonsense transcripts with genomic mutations. Many versions of the NMD microarray technology have been created, including incubations with emetine only, emetine together with or followed by actinomycin D, as well as RNA interference treatment to silence a key NMD component, such as Rent1. We have advocated the integration with CGH array data as well as kinetic assays to determine transcript decay rates, and to increase the signal relative to noise. The optimal method to achieve detection of transcript decay rates remains to be determined. In our previous study [11], actinomycin D treatment was applied to stop transcription of the cancer cells, thereby allowing changes in decay rates to be better defined. We have recently observed (unpublished data of the authors) that the application of actinomycin D after emetine incubation does not have a significant effect on the treated cells, suggesting that this combination of the two drugs may not work as well as initially suggested. This is most likely due to the non-specific stabilization of the transcriptome, which has been suggested to be induced by most translational inhibitors (such as emetine), thereby protecting the transcripts from the degradation machinery [32]. Therefore, rather than using the chemical translation inhibitors, RNAi-based knockdown of specific components of the NMD machinery may be more informative.

It is important to note that the NMD-based methods are research tools for cancer gene discovery and are applied in cancer cell lines grown *in vitro*, not for human tumor diagnosis. It is generally known that such *in vitro* growing cancer cell lines contain additional genetic alterations unrelated to tumorigenesis, making verification of the initial findings in patient samples crucial in the molecular pathology laboratories. In the future, it may be possible to optimize the method for studying patient samples, such as lymphoblastoid cell lines from patients with hereditary cancer, or in primary cultures of cancer cells (such as leukemias).

In summary, microarray-based NMD inhibition survey holds great potential as a rapid discovery tool for identifying nonsense-mutated transcripts in a whole-genome screening assay, but the optimal strategy for NMD manipulation remains to be determined.

References

[1] E. Batlle, J.T. Henderson, H. Beghtel, M.M.W. van den Born, E. Sancho, G. Huls, J. Meeldijk, J. Robertson, M. van de Wetering, T. Pawson and H. Clevers, β-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/EphrinB, *Cell* **111** (2002), 251–263.

[2] P. Belgrader, J. Cheng, X. Zhou, L.S. Stephenson and L.E. Maquat, Mammalian nonsense codons can be *cis* effectors of nuclear mRNA half-life, *Mol. Cell. Biol.* **14** (1994), 8219–8228.

[3] J. Cheng, P. Belgrader, X. Zhou and L.E. Maquat, Introns are *cis* effectors of the nonsense-codon-mediated reduction in nuclear mRNA abundance, *Mol. Cell. Biol.* **14** (1994), 6317–6325.

[4] E. Conti and E. Izaurralde, Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species, *Curr. Opin. Cell. Biol.* **17** (2005), 316–325.

[5] G. Denning, L. Jamieson, L.E. Maquat, E.A. Thompson and A.P. Fields, Cloning of a novel phosphatidylinositol kinase-related kinase: characterization of the human SMG-1 RNA surveillance protein, *J. Biol. Chem.* **276** (2001), 22709–22714.

[6] J.-T. Dong, Chromosomal deletions and tumor suppressor genes in prostate cancer, *Cancer Metastasis Rev.* **20** (2002), 173–193.

[7] P.A. Frischmeyer and H.C. Dietz, Nonsense-mediated mRNA decay in health and disease, *Hum. Mol. Genet.* **8** (1999), 1893–1900.

[8] P.A. Futreal, L. Coin, M. Marshall, T. Down, T. Hubbard, R. Wooster, N. Rahman and M.R. Stratton, A census of human cancer genes, *Nat. Rev. Cancer* **4** (2004), 177–183.

[9] J.T. Henderson, J. Georgiou, Z. Jia, J. Robertson, S. Elowe, J.C. Roder and T. Pawson, The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function, *Neuron* **32** (2001), 1041–1056.

[10] S.P. Hussain, L.J. Hofseth and C.C. Harris, Tumor suppressor genes: at the crossroads of molecular carcinogenesis, molecular epidemiology and human risk assessment, *Lung Cancer* **34**(Suppl.) (2001), S7–S15.

[11] P. Huusko, D. Ponciano-Jackson, M. Wolf, J.A. Kiefer, D.O. Azorsa, S. Tuzmen, D. Weaver, C. Robbins, T. Moses, M. Allinen, S. Hautaniemi, Y. Chen, A. Elkahloun, M. Basik, G.S. Bova, L. Bubendorf, A. Lugli, G. Sauter, J. Schleutker, H. Ozcelik, S. Elowe, T. Pawson, J. Trent, J.D. Carpten, O.-P. Kallioniemi and S. Mousses, Nonsense-mediated decay microarray analysis identifies mutations of *EPHB2* in human prostate cancer, *Nat. Genet.* **36** (2004), 979–983.

[12] Â. Inácio, A.L. Silva, J. Pinto, X. Ji, A. Morgado, F. Almeida, P. Faustino, J. Lavinha, S.A. Liebhaber and L. Romão, Nonsense mutations in close proximity to the initiation codon fail to trigger full nonsense-mediated mRNA decay, *J. Biol. Chem.* **279** (2004), 32170–32180.

[13] Y. Ionov, N. Nowak, M. Perucho, S. Markowitz and J.K. Cowell, Manipulation of nonsense mediated deacy identifies gene mutations in colon cancer cells with microsatellite instability, *Oncogene* **23** (2004), 639–645.

[14] Y. Ionov, S.-I. Matsui and J.K. Cowell, A role for p300/CREB binding protein genes in promoting cancer progression in colon cancer cell lines with microsatellite instability, *Proc. Natl. Acad. Sci. USA* **101** (2004), 1273–1278.

[15] A.G. Knudson, Jr., Hereditary cancer, oncogenes, and antioncogenes, *Cancer Res.* **45** (1985), 1437–1443.

[16] K. Kullander and R. Klein, Mechanisms and functions of Eph and ephrin signalling, *Nat. Rev. Mol. Cell. Biol.* **3** (2002), 475–486.

[17] H. Le Hir, E. Izaurralde, L.E. Maquat and M.J. Moore, The spliceosome deposits multiple proteins 20–24 nucleotides upstream of mRNA exon–exon junctions, *EMBO J.* **19** (2000), 6860–6869.

[18] F. Lejeune, X. Li and L.E. Maquat, Nonsense-mediated mRNA decay in mammalian cells involved decapping, deadenylation, and exonucleolytic activities, *Mol. Cell* **12** (2003), 675–687.

[19] J.E. Lew, S. Enomoto and J. Berman, Telomere length regulation and telomeric chromatin require the nonsense-mediated mRNA decay pathway, *Mol. Cell. Biol.* **18** (1998), 6121–6130.

[20] B.P. Lewis, R.E. Green and S.E. Brenner, Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans, *Proc. Natl. Acad. Sci. USA* **100** (2003), 189–192.

[21] R. Losson and F. Lacroute, Interference of nonsense mutations with eukaryotic messenger RNA stability, *Proc. Natl. Acad. Sci. USA* **76** (1979), 5134–5137.

[22] J. Lykke-Andersen, M.-D. Shu and J.A. Steitz, Communication of the position of exon–exon junctions to the mRNA surveillance machinery by the protein RNPS1, *Science* **293** (2001), 1836–1839.

[23] K. Macleod, Tumor suppressor genes, *Curr. Opin. Genet. Dev.* **10** (2000), 81–93.

[24] L.E. Maquat, Nonsense-mediated mRNA decay in mammals, *J. Cell. Sci.*, **118** (2005), 1773–1776.

[25] L.E. Maquat, Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics, *Nat. Rev. Mol. Cell. Biol.* **5** (2004), 89–99.

[26] J.T. Mendell, N.A. Sharifi, J.L. Meyers, F. Martinez-Murillo and H.C. Dietz, Nonsense surveillance regulates expression of diverse classes of mammalian transcripts and mutes genomic noise, *Nat. Genet.* **36** (2004), 1073–1078.

[27] P.M. Moriarty, C.C. Reddy and L.E. Maquat, Selenium deficiency reduces the abundance of mRNA for Se-dependent glutathione peroxidase 1 by UGA-dependent mechanism likely to be nonsense codon-mediated decay of cytoplasmic mRNA, *Mol. Cell. Biol.* **18** (1998), 2932–2939.

[28] E. Nagy and L.E. Maquat, A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. *Trends Biochem. Sci.* **23** (1998), 198–199.

[29] E.N. Noensie and H.C. Dietz, A strategy for disease gene identification through nonsense-mediated mRNA decay inhibition, *Nat. Biotech.* **19** (2001), 434–439.

[30] C.C. Oliveira and J.E.G. McCarthy, The relationship between eukaryotic translation and mRNA stability, *J. Biol. Chem.* **270** (1995), 8936–8943.

[31] R. Parker and H. Song, The enzymes and control of eukaryotic mRNA turnover, *Nat. Struct. Mol. Biol*. **11** (2004), 121–127.

[32] J. Ross, A hypothesis to explain why translation inhibitors stabilize mRNAs in mammalian cells: mRNA stability and mitosis, *Bioessays* **19** (1997), 527–529.

[33] B. Vogelstein and K.W. Kinzler, Cancer genes and the pathways they control, *Nat. Med.* **10** (2004), 789–799.

[34] G.M. Wilson, Y. Sun, J. Sellers, H. Lu, N. Penkar, G. Dillard and G. Brewer, Regulation of AUF1 expression via conserved alternatively spliced elements in the $3'$ untranslated region, *Mol. Cell. Biol.* **19** (1999), 4056–4064.

[35] A. Yamashita, T. Ohnishi, I. Kashima, Y. Taya and S. Ohno, Human SMG-1, a novel phosphatidylinositol 3-kinase-related protein kinase, associates with components of the mRNA surveillance complex and is involved in the regulation of nonsense-mediated mRNA decay, *Genes. Dev.* **15** (2001), 2215–2228.