# Detection of intronic polymorphism in heterozygous ductal breast carcinoma samples by non-isotopic RNase Cleavage Assay

**Nordin N[1], Thilakavathy K[1], Seong NK[2], Sabariah AR[2] and Rosli R[1]** *[1]Department of Human Growth and Development, [2]Department of Clinical Laboratory Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia. (Correspondence: Asssoc Prof Dr Rozita Rosli; e-mail: rozita@medic.upm.edu.my)*

**Abstract**

Alterations of *p53* gene, a tumour-suppressor gene in various human malignancies, have been well characterised in half of human cancers, with 10-30% alterations in breast carcinoma cases. An established screening method is important in order to reveal the *p53* mutation patterns to strengthen its value as a predictive and prognostic marker in breast cancer. However, the wide variation in quality and quantity of the genomic DNA derived from clinical samples has often challenged the mutation detection strategies. Therefore, a reliable and sensitive detection method is needed in order to overcome the problem as well as to detect the various types of mutations scattered over the coding exons, especially exons 5-8, and their flanking intron sequences. The present study has managed to establish a mutation screening method using a non-isotopic RNase Cleavage Assay (NIRCA™) from thirty-one invasive ductal breast carcinoma samples for intron 4 through exon 9 of the *p53* gene. DNA sequence analysis was conducted to confirm the mutation spots screened by NIRCA™. Interestingly, all samples studied showed intronic alterations with no mutation detected in the mutation hotspots of exons 5-8. Two intronic polymorphisms, as described elsewhere, were robustly detected in almost 50% of the samples screened by this method. This finding is important to our understanding of the biology of breast cancer and is an important contribution to the establishment of a Malaysian *p53* mutation database.

**Key words:** Intronic mutation, *p53*, invasive ductal breast carcinoma, heterozygosity

## Introduction

Breast cancer is the most common cancer in women with an increase of 33% incidence rate in 10 years (Boyle, 1997) and is the leading cause of mortality in women. One approach that may advance the understanding of the increased incidence of breast cancer is to study the *p53* tumour suppressor gene, which orchestrates cell cycle control, DNA repair, apoptosis and control of angiogenesis (Phillips, 1999; Bellamy, 1997; Wynford, 1996). Other common genes implicated in breast cancer are *BRCA-1, BRCA-2, erbB2 (HER-2, neu)* and *ras*. Together with *ras* and *c-erB-2* genes, alteration of the *p53* gene during carcinogenesis is suspected to be activated early in the development of breast cancer (Duffy, 1995). The *p53* protein has 3 domains: the amino-terminal domain is involved in transcriptional activation, the central or core domain is responsible for sequence-specific binding of the protein to DNA and finally, the carboxy-terminal domain contains nuclear localization signals, DNA damage recognition sites and protein oligomerization sites (Phillips, 1999). Obviously, the *p53* gene is important in cellular homeostasis and function. Thus, the high frequency of mutations of this tumour suppressor gene in human cancer (mutations are found in over 50% of human cancers) reflects the importance of its involvement in maintaining the stability of the genome during cellular stress (Benette *et al.*, 1999). This is the reason why the gene is best suited for mutational spectrum analysis besides its modest size (11 exons, 393 amino acids) which allows for the study of the entire coding region. The presence of *p53* mutations in human cancers might be of value as a molecular marker for early prognosis especially in breast cancer cases since mutation in *p53* results in poor prognosis, weak response to therapy and decreased overall survival time (Benette *et al.*, 1999).

Most common *p53* mutations are found in both familial and sporadic breast cancers (Coles *et al.*, 1992; Greenblatt *et al.*, 1994). However, the majority of mutations in human cancers are somatic rather than germline. Approximately, 80% of the *p53* mutations are misssense point mutations, mostly concentrated within the central DNA binding region (codon 110-307) (Phillips,

1999). In breast cancer cases, many investigations have concluded that the mutations are more common in invasive ductal breast carcinoma than in lobular cancers with exons 5-8 being the mutation hotspots. However, mutations lying outside this region or in intronic sequences may be significant and have been previously underestimated (Phillips, 1999). It has been suggested that up to 25% of *p53* mutations are located outside exons 5-8 (Thompson-Hehir *et al.*, 2000). Previous *p53* mutation studies on breast cancer and other type of cancers have identified intronic mutations to be frequently located at the splice sites within introns 3, 4, 7 and 9 (Thompson-Heir *et al.*, 2000; Pollett *et al.*, 2000; Bromidge *et al.*, 2000). It is therefore important to determine the nature and location of *p53* mutations discovered in a particular disease as it may reflect the causative mutagenic insults (Phillips, 1999).

Presently, most reported studies on mutations of *p53* especially in breast cancer concentrate on the immunohistochemical analysis of *p53* expression from paraffin embedded tissues (Lukas *et al.*, 2000). In this study, a molecular screening technique was applied and the *p53* gene was screened for potential mutations in intron 4 through exon 9 using a non-isotopic RNase Cleavage Assay (NIRCA) analysis. The results of the study show that successful amplification of the region in three fragments of the *p53* gene from genomic DNA extracted from thirty-one paraffin-embedded breast cancer samples using two rounds of polymerase chain reaction (PCR) was achieved. The exact location of the mutations was determined by DNA sequence analysis. The study found NIRCA to be rapid, and suitable for use as a high-throughput genetic screening method for detection of mutations in the *p53* gene.

## Material and Methods

*Samples*

Breast tissues processed as paraffin-embedded samples obtained in duplicated sets from Subang Jaya Medical Center and Specialty Laboratories Klang, Malaysia were randomly selected from patients with invasive ductal breast carcinoma aged from 18 to 62 years old. The tissues were then stained with hematoxylin and eosin (H&E) for identification of the cancerous region. The identification of cancerous region(s) was confirmed by a pathologist. A total of thirty-one samples with cancerous regions were then subjected to DNA extraction.

*DNA extraction*:

Based on the identification of the cancerous region on H&E stained slides, DNA extraction from duplicate paraffin-embedded slides were conducted. Only the identified location on each slide was extracted using the protocols recommended by the manufacturer (Pin-Point DNA Extraction Kit, Zymo Research).

*p53 amplification*:

The extracted DNA from the identified regions was then subjected to two rounds of polymerase chain reaction (PCR) to amplify exons 5-9. Three sets of outer primers (for first round PCR, Ambion) and three sets of nested primers (T7 and T7 promoter-incorporated primers for second round of PCR, Ambion) were used to amplify the target regions (exons 5-6, 7 and 8-9).

*Amplification of exons 5-9*:

The PCR was performed using a Mastercycler Gradient (Eppendorf) to optimize the conditions since different annealing temperatures were needed for different sets of primers. The same PCR conditions were applied for both rounds of PCR. For each reaction sample, 50-150 ng of genomic DNA was mixed with 5mM of each primers (Ambion), 1.9 mM of $Mg^{++}$, 0.2mM of each dNTPs, and 2.5U of Taq polymerase (Promega) in total reaction of 30 µl. The reaction was heated at 95°C for 5 minutes prior to addition of the enzyme. The PCR parameters used were denaturing at 95°C for 40 seconds, annealing at 61°C for fragment II, 59°C for fragment I, and 57°C for fragment III; for 40 seconds, and extension at 72°C for 50 seconds. The parameters were repeated for 30 cycles.

*Non-Isotopic RNase Cleavage Assay (NIRCA, Ambion)*

Transcription of RNA was carried out by adding 50-150 ng of the PCR product into 4 µl of transcription assay following the protocol suggested by the manufacturer (Mutation Screener™, Ambion). Hybridization solution was added into the reaction mixture prior to denaturation of the RNA duplexes at 97°C for 7 minutes (Mastercycle Gradient, Eppendorf). The hybridization of the denatured RNA transcripts was then carried out at room temperature for 5 minutes followed by cleavage of RNA mismatches by RNase I and T1 (Ambion) digestion at 37°C for 35 minutes. The digestion reaction was stopped by the addition of stop dye

solution (Ambion). The digested fragments were then analysed on a 2.5% ethidium bromide-free agarose gel. The electrophoresis was conducted in 1X TBE buffer at 130 V for two hours.

*DNA Sequencing Analysis*

All fragments showing alteration of the *p53* gene and one of each fragment showing no alteration of the gene by NIRCA were sequenced to confirm the reliability of NIRCA and also to determine the exact location of the mutations.

**Results**

In order to perform the NIRCA, T7-T7 promoters incorporated PCR product is needed. We successfully amplified the three fragments of sizes 600 bp, 406 bp and 411 bp of fragment I, fragment II and fragment III of the *p53* gene, respectively (Fig. 1) from paraffin-embedded breast tissues. Fragment I consists of 150 bases of intron 4, the whole of exon 5, intron 5 and exon 6, and 130 bases of intron 6. Fragment II amplified 70 bases of intron 6, the whole of exon 7 and 220 bases of intron 7. Fragment III contains 100 bases of intron 7, the whole of exon 8, intron 8 and exon 9, and 70 bases of intron 9. The presence of RNA transcripts was identified by an increase of ethidium bromide stained material in the transcription assay as compared to the intensity of PCR product (Fig. 2). Samples were classified as having the mismatches when a smaller fragment(s) than the size of the PCR product was produced after RNase digestion (Fig. 3). The 31 breast cancer samples were screened and all showed potential mutation spots by NIRCA. The mismatch(s) was found in only fragment II of the *p53* gene (Figs. 3a, 3b, 3c, & 3d) while no alteration was detected when samples from non-cancerous tissue were screened.

We have categorised NIRCA results into four groups based on the pattern and size of the cleavage products after RNase digestion. It is important to estimate that the newly observed cleavage products equals the observed length of the full-length double-stranded RNA, even though they do not exactly co-migrate with the standard DNA markers (Prosser & Condie, 1991). Based on these NIRCA results, at least three different sites of alteration were expected. Interestingly, sequencing analysis confirmed the reliability of NIRCA by detecting mismatch(es) in all potential mutation spot(s) screened by NIRCA (Figs. 4a & 4b) and revealed two intronic polymorphisms that exactly matched the ones described in the *p53* database (Fig. 4b). Group 1 consists of twelve samples, which have transversions of T to C and T to G at nucleotide 2682 and 2702, respectively. The digestion produced two digested fragments of sizes ~300 bp and >100 bp (Fig. 3a). The other expected band of size 20 bp was not observed as such a


Figure 1. Nested PCR products. Lanes 1-7 show the PCR products of size 600 bp for fragment I. Lanes 8-14 show the PCR products of size 406 bp for fragment II. Lanes 15-21 show the PCR products of size 411 bp for fragment III.

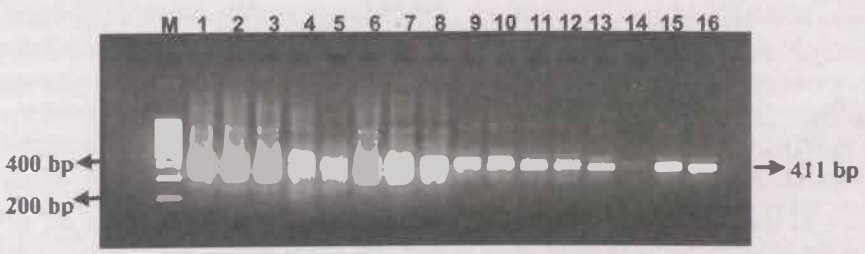


Figure 2. Transcription assay. Lanes 1-8 show the transcription assay from PCR products of fragment III. Lanes 9-16 show the PCR products of size 411 bp (fragment II) from which the transcripts were made.

small product is difficult to visualise. Group 2 consists of four samples with a transversion of T to C at nucleotide 2682 and a deletion of G at nucleotide 2470 (Figs. 3b, 4ai & 4bi). The digestion produced one cleavage product of expected size >300 bp, and two invisible small products. Groups 3 and 4 consist of five samples having a transversion of T to G at nucleotide 2702 and ten samples with a transversion of T to C at nucleotide 2682, respectively. All the five samples exhibit an expected digested fragments of ~300 bp and one invisible small fragment (Fig. 3c), while group 4 samples show one expected band of size <300 bp and one small invisible small fragment (Fig. 3d).

**Discussion**

Presently, there are no confirmed prognostic variables yet established for early diagnosis of breast cancer. Diagnosis for breast cancer was established based on combination of traditional clinical staging (such as local extension, size, and presence or absence of nodal or distant metastases), standard histological analysis of the tumour specimen, and expression of hormone receptor in the tumour (Clark *et al.*, 2000). Thus, most cases of breast cancer patients present with late stage of the tumour. However, recent studies on the genetic composition of the cancer cell and the availability of molecular biological probes in detecting variations in oncogene and other related genes expression have revealed several putative prognostic factors in breast cancer (Clark *et al.*, 2000). Therefore, early detection of cancer, cancer cure and prevention may soon be possible.

In this study, NIRCA was used to detect the *p53* mutations. This method has been shown to have excellent performance characteristics where it can directly detect mutations or other sequence polymorphisms with relatively low false positive and false negative results. Alteration or the mismatch detected by NIRCA is less likely to be caused by the two rounds of PCR as the negative control does not exhibit any mismatch. The method produced no false positive in the cloning and sequencing of over seventy *p53* gene mutations (Prescott *et al.*, 1999). Sequencing analysis on all samples with NIRCA potential mutation spots and twelve samples showing no potential mutation spots confirmed the reliability of NIRCA in screening for mutation. NIRCA can also be used in detecting mutations in homozygous as well as heterozygous samples. The sequencing results have confirmed our heterozygous samples as the mismatch spot showed double peaks. Previous studies have proven the method to be a sensitive, non-radioactive, and high-throughput genetic screener (Goldrick *et al.*, 1996). NIRCA has advantages over other mutation detection methods such as direct DNA sequence determination, SSCP analysis, LOH analysis and DGGE, as the specific cleavage products can be observed easily and it can analyse relatively large fragments of DNA in a single reaction (Prescott *et al.*, 1999). This mutation detection strategy has been applied efficiently not only in breast cancer, but also in bladder, colon and lung cancers (Prescott *et al.*, 1999).

In the present study, we have optimised the DNA extraction from paraffin-embedded breast cancer samples and the NIRCA protocols for screening and identifying the location for the point mutations. The present work reveals the first intronic polymorphisms among 31 invasive ductal carcinoma cases in Malaysia. Our results have identified two intronic polymorphisms within intron 7, which are transversion of T to G at nucleotide 2702 (55% occurrence) and transversion of T to C at nucleotide 2682 (84% occurrence). These two polymorphisms were also described by Prosser & Condie (1991), and Berggren *et al.* (2001) using different detection methods. Presently, more studies have been conducted on the intronic polymorphisms and its significance towards the development of tumorigenesis has not been underestimated. A recent study showed intronic polymorphism in the *p53* gene increases breast cancer risk appreciably in women by the age of 50 years with a family history of breast cancer in the German population (Wang-Gohrke *et al.*, 2002). Thompson-Hehir *et al.* (2000) identified a T-to-G base change within intron 7 (12 bases away from our spot) in their study on ovarian cancer. Intronic *p53* changes were also detected in breast cancer where the alterations were located within introns 6 and 9 (Pollett *et al.*, 2000). Voglino *et al.* (1997) found a deletion of 36 bp in intron 5 resulting in exon 6 skipping in breast cancer samples. *p53* intronic mutations have also been observed in many cancer cases such as Li-Fraument syndrome (Varley *et al.*, 1998), hepatocellular carcinoma (Lai *et al.*, 1993), lung

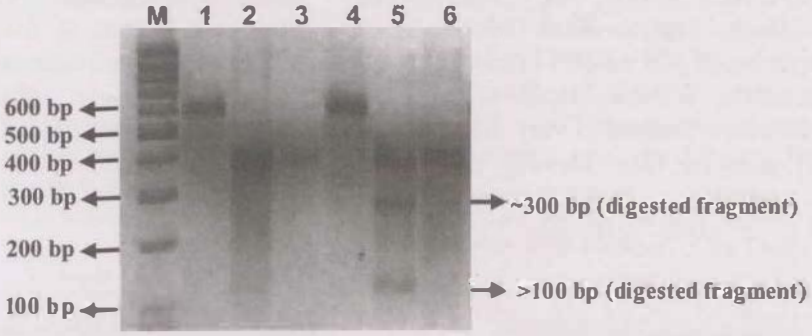


Fig. 3a

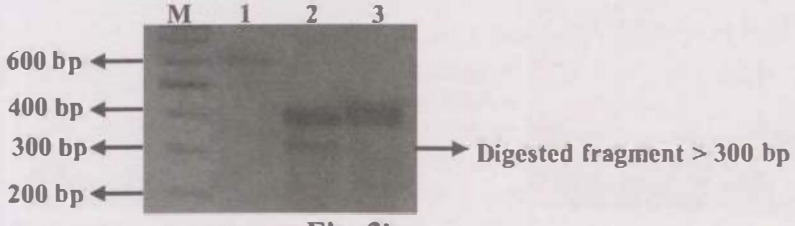


Fig. 3b

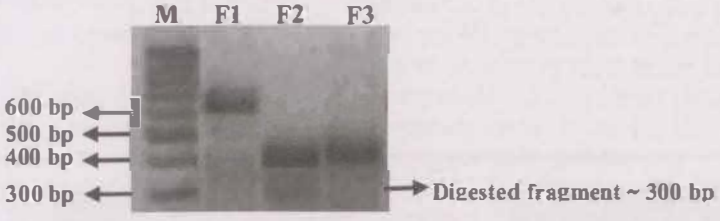


Fig. 3c

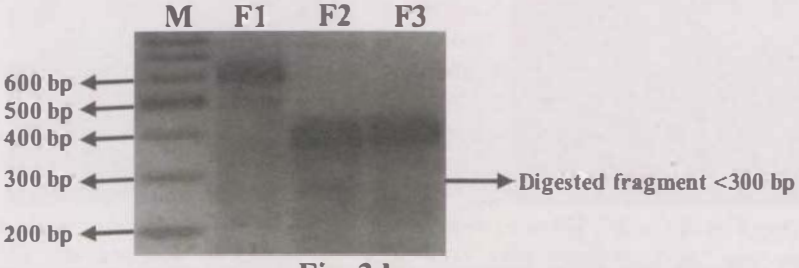


Fig. 3d

**Figure 3**. RNAse digestion. The mismatch was identified by the presence of smaller digested fragments (>100 bp, >300 bp, <300 bp, and ~300 bp) as compared to the original RNA duplexes (about the same size of the PCR products). **(3a)** Lanes 1-3 represent the three fragments of the RNA heteroduplexes amplified from DNA extracted from a non-cancerous tissue showing no digested fragment when the duplexes were digested with RNAse. Lanes 4 and 6 show that no mismatch was detected in fragments I and III, respectively. Lane 5 shows the digested fragments of ~300 bp and >100 bp within fragment II. **(3b)** Lanes 1 and 3 show that no mismatch is detected in fragments I and III, respectively. Lane 2 shows the digested fragment (>300 bp) within fragment II (lane 2). **(3c)** Lanes F1 and F3 show that no mismatch is detected in fragments I and III, respectively. Lane F2 shows a digested fragment of size ~300 bp within fragment II. **(3d)** Lanes F1 and F3 show that no mismatch is detected in fragments I and III, respectively. Lane F2 shows a digested fragment of size <300 bp within fragment II.

cancer cell lines (Takahashi *et al.*, 1990), myelodysplastic syndrome (Kikukawa *et al.*, 1998) and B-chronic lymphocytic leukaemia (Bromidge *et al.*, 2000), where all mutations detected affected splice sites which led to aberrant splicing of *p53* mRNA in all cases.

An accurate, reliable, rapid and efficient mutation detection method is very crucial as *p53* mutational analysis may identify patients for effective treatments. NIRCA may have the potential as a rapid screening tool for *p53* mutations of breast and other cancers, especially those with a family history. The information on the mutation analysis and polymorphisms would contribute significantly to a database of mutations unique to the Malaysian population. This would provide information needed to initiate work on pharmacogenomics, gene therapy and cancer vaccine. The analysis would be able to identify family members who are at risk of developing cancer for genetic counselling.

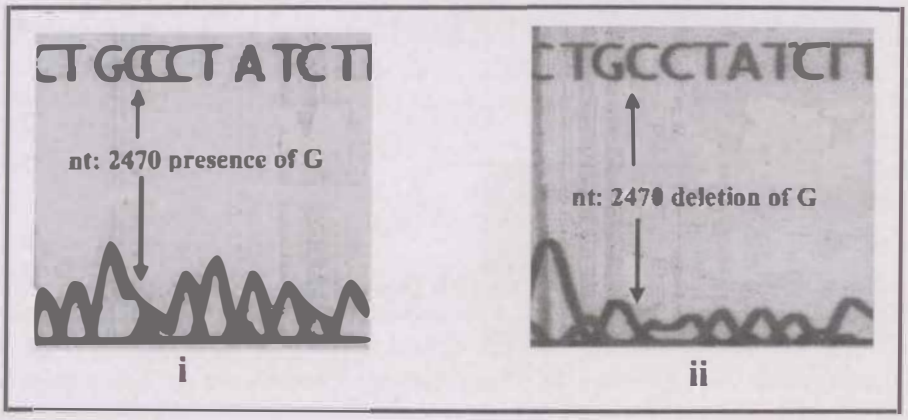


**Fig. 4a**

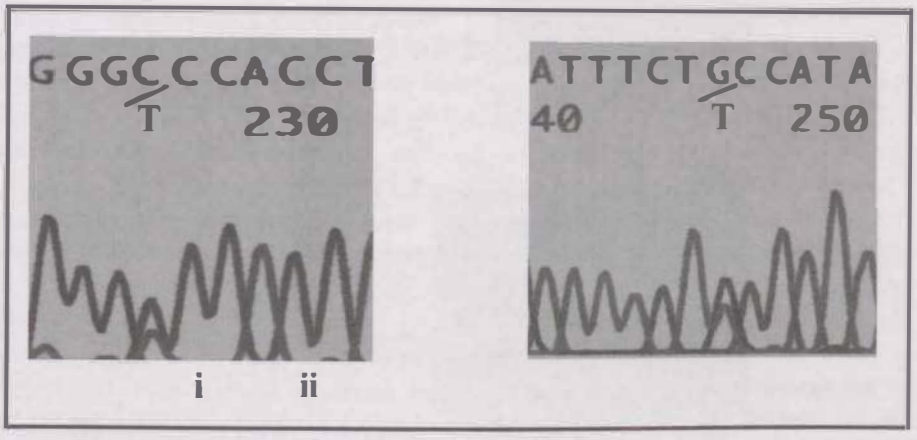


**Fig. 4b**

**Figure 4.** Sequence analysis. Sequencing results confirmed the mismatches that were detected by NIRCA. **(4ai)** No deletion was detected in samples without digested fragments; **(4aii)** A deletion of G at nucleotide 2470 was identified for samples with digested fragments of >300 bp; **(4bi)** A polymorphism of T to C was detected at nucleotide 2682. Double peaks for the mismatches indicate that the samples used were heterozygous; **(4bii)** A polymorphism of T to G was found at nucleotide 2702 when samples with digested fragments of ~300 bp and >100 bp, and ~300 bp. Double peaks for the mismatches indicate that the samples used were heterozygous.

**Acknowledgement**

This project is supported by IRPA grant No. 06-02-04-0017 from the Ministry of Science, Technology and the Environment, Malaysia. We also would like to thank Dr. Marianna M Goldrick for her helpful discussion and assistance.

References

Andersen TI, Holm R, Nesland JM, Heimdal KR, Ottestad L, Borresen A. Prognostic significance of TP53 alterations in breast carcinoma. *Br J Cancer* 1993, 68: 540-8.

Bellamy CO. p53 and apoptosis. *Br Med Bull* 1997, 53: 532-8.

Bennett WP, Hussain SP, Vahakangas KH, Khan MA, Shields PG, Harris CC. Molecular epidemiology of human cancer risk: Gene-environment interactions and p53 mutation spectrum in human lung cancer. *J Pathol* 1999, 187: 8-18.

Berggren P, Kumar R, Steinede G, Ichiba M, Hemminki K. Ethnic variation in genotype frequencies of a p53 intron 7 polymorphism. *Mutagenesis* 2001, 16(6): 475-8.

Berns EMJ, Van Staveren IL, Look MP, Smid M, Klijn JMG, Foekens JA. Mutations in residues of TP53 that directly contact DNA predict poor outcome in human primary breast cancer. *Br J Cancer* 1998, 77: 1130-6.

Borresen A, Andersen TI, Eyfjord JE, Cornelis RS, Thorlacius S, Borg A, Johansson U, Theillet C, Scherneck S, Hartman S. TP53 mutations and breast cancer prognosis particularly poor survival rates for cases with mutations in the zinc-binding domains. *Genes Chromosomes Cancer* 1995, 14(1): 71-5.

Boyle P. Global burden of cancer. *Lancet* 1997, 349(suppl II): 23-6.

Bromidge T, Lowe C, Prentice A, Johnson S. p53 intronic point mutation, aberrant splicing and telomeric associations in a case of B-chronic lymphocytic leukemia. *Br J Haematology* 2000, 111: 223-9.

Clark JL, Nguyen PL, Jaszcz WB, Jatoi A. Prognostic variables in male breast cancer. *The American Surgeon* 2000, 66: 502-10.

Coles C, Condie A, Chetty U, Steel CM, Evans HJ, Prosser J. p53 mutations in breast cancer. *Cancer Res* 1992, 52(19): 5291.

Lai MY, Chang HC, Li HP, Ku CK, Chen PJ, Sheu JC, Huang GT, Lee PH, Chen DS. Splicing mutations of the p53 gene in human hepatocellular carcinoma. *Cancer Res* 1993, 53: 1653-6.

Duffy MJ. Can molecular markers now be used for early diagnosis of malignancy? *Clin Chem* 1995, 41(10): 1410-3.

Foti A, Bar-Eli M, Ahuja HG, Cline MJ. A Splicing mutation accounts for the lack of p53 gene expression in a CML blast crisis cell line: a novel mechanism of p53 gene inactivation. *Br J Haematology* 1990, 76: 143-5.

Goldrick MM, Kimball GR, Liu Q, Martin LA, Sommer SS, Tseng JY. NIRCA: a rapid robust method for sequencing for unknown point mutations. *Biotechniques* 1996, 21(1): 106-12.

Greenblatt MS, Bennett WP, Hollstein M, Harris C. Mutations in the p53 tumour suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994, 54: 4855.

Kikukawa M, Aoki N, Mori M. A case of myelodysplastic syndrome with an intronic point mutation of the p53 suppressor gene at the splice donor site. *Br J Haematol* 1998, 100: 564-6.

Lai M-Y, Chang H-C, Li H-P, Ku C-K, Chen P-J, Sheu J-C, Huang G-T, Lee P-H, Chen Lukas J, Niu N, Press MF. p53 mutations and expression in breast carcinoma in situ. *Am J Pathol* 2000, 156(1): 183-90.

Phillips HA. The role of the p53 tumour suppressor gene in human breast cancer. *Clin Oncol* 1999, 11: 148-55.

Pollett A, Bedard YC, Li SQ, Rohan T, Kandel R (2000) Correlation of p53 mutations in thinprep-processed fine needle breast aspirates with surgical resected breast cancers. Mod Pathol 13(11): 1173-9.

Prescott J, Patel H, Tilman S, McHugh T, Ralph, D. Cleavage of double-stranded copy RNA by RNase 1 and RNAse TI provides a robust means to detect p53 gene mutations in clinical specimens. *Electrophoresis* 1999, 20(6): 1149-61.

Prosser J, Condie A. Biallelic Apa 1 polymorphism of the human p53 gene. *Nucleic Acid Res* 1991, 19(17): 4799.

Takahashi T, D'Amico D, Chiba I, Buchhagen DL, Minna JD. Identification of intronic point mutations as an alternative mechanism for p53 inactivation in lung cancer. *J Clin Invest* 1990, 86: 363-9.

Thompson-Hehir J, Davies MP, Green JA, Halliwell N, Joyce KA, Salisbury J, Sibson DR, Vergote I Walker C. Novel polymerase chain reaction approach for full-coding p53 mutation detection in microdissected archival tumours. *Diagn Mol Pathol* 2000, 9(2): 110-9.

Thorlacius S, Borresen AL, Eyfjord JE. Somatic p53 mutations in human breast carcinomas in an Icelandic population: a prognostic factor. *Cancer Res* 1993, 53: 1637-41.

Van Slooten H, Van De Vijver MJ, Borresen A-L, Eyejord JE, Valgardsdottir R, Scherneck S, Nesland JM, Devilef P, Cornelissf CJ, Van Dierendonck JH. Mutations in exons 5-8 of the p53 gene, independent of their type and location, are associated with increased apoptosis and mitosis in invasive breast carcinoma. *J Pathol* 1999, 189: 504-13.

Varley JM, Thorncroft M, White GRM, Tricker KJ, Kelsey AM, Birch JM, Evans DG. A novel TP53 splicing mutation in a Li-Fraumeni syndrome family: a patient with Wilms' tumour is not a mutation carrier. *Br J Cancer* 1998, 78: 1081-3.

Voglino G, Castello S, Silengo L, Stefanuto G, Friard O, Ferrara G, Fessia L. An intronic deletion in TP53 gene causes exon 6 skipping in breast cancer. *Eur J Cancer* 1997, 33(9): 1479-83.

Wang-Gohrke S, Becher H, Kreienberg R, Runnebaum IB, Chang-Claude J. Intron 3 16 bp duplication polymorphism of p53 is associated with

an increased risk fro breast cancer by the age of 50 years. *Pharmacogenetics* 2002, 12(3): 269-72

Wynford TD (1996) p53: Guardian of cellular scenescence. *J Pathol* 1996, 180: 118-21.