Copy-number variation of MCL1 predicts overall survival of non-small-cell lung cancer in a Southern Chinese population

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Keywords
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Abstract
BCL2L1 and MCL1 are key anti-apoptotic genes, and critical for cancer progression. The prognostic values of BCL2L1 and MCL1 copy-number variations (CNVs) in non-small-cell lung cancer (NSCLC) remain largely unknown. Somatic CNVs in BCL2L1 and MCL1 genes were tested in tumor tissues from 516 NSCLC patients in southern China; afterward, survival analyses were conducted with overall survival (OS) as outcome. Additionally, the associations between CNVs and mRNA expression levels were explored using data from 986 NSCLC patients in the Cancer Genome Atlas project. It was found that amplifications of BCL2L1 and MCL1 were associated with unfavorable OS of NSCLC, with adjusted hazards ratio of 1.62 (95% confident interval [CI] = 1.10–2.40; \( P = 0.015 \)) and 1.39 (95% CI = 1.05–1.84; \( P = 0.020 \)), respectively. Amplifications of MCL1, but not BCL2L1, were related with higher mRNA expression levels of corresponding gene, compared with non-amplifications (\( P = 0.005 \)). Interestingly, after incorporating with MCL1 CNV status, clinical variables (age, sex, TNM stage, and surgical approach) showed an improved discriminatory ability to classify OS (area under curve increased from 72.2% to 74.1%; \( P = 0.042 \), DeLong’s test). Overall, MCL1 CNV might be a prognostic biomarker for NSCLC, and additional investigations are needed to validate our findings.

Introduction
An estimated 1.8 million new lung cancer cases occurred in 2012, accounting for about 13% of total cancer diagnoses [1]. Lung cancer was the most frequently diagnosed cancer and the leading cause of cancer death among males in 2012. Among females, lung cancer was the leading cause of cancer death in more developed countries, and the second leading cause of cancer death in less developed countries [1]. In 2013, lung cancer was the fourth most common cause of death for men and women in China overall [2], and lung cancer is the number one cause of death among people with malignant tumors in China [3]. Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer. Although treatment (such as surgery, radiotherapy, and/or chemotherapy) has improved the rate of survival of patients with NSCLC in recent years, the long-term survival rate still has room to improve. TNM
MCL1 proposed that the anti-apoptotic Bcl-2 family members play an important role in resisting cell death but also has functional roles in many cancers, including lung cancer, where ROS1 rearrangements, MET amplification, RET fusions, and activating mutations in BRAF, HER2, and KRAS in frequencies exceeding 1% [5]. Nevertheless, it is possible that some other unknown genetic factors may also modulate survival outcomes of NSCLC patients.

Studies suggest that Bcl family not only plays an important role in resisting cell death but also has functional roles in cell-cycle control [6]. At the same time, it is proposed that the anti-apoptotic Bcl-2 family members may participate in the inhibition of autophagy, which is believed to be a nonapoptotic form of programmed cell death [7]. Altered expression of members in this family leads to aberrant cell proliferation and malignant growth [8, 9]. Study also supports the idea that Bcl family proteins represent important therapeutic targets [10]. BCL2L1, also known as BCL-X, is mapped to chromosome 20. Amplifications of 20q have been previously noted in various cancers [11, 12]. BCL2L1 proteins regulate outer mitochondrial membrane channel opening, which in turn regulates mitochondrial membrane potential, and thus controls the release of cytochrome and the production of reactive oxygen species, both of which are potent inducers of programmed cell death [13]. It is shown that BCL2L1, but not BCL2, could suppress mitophagy mediated by FUNDC1 through its BH3 domain, eventually mediating mitochondrial quality control that is essential for cell survival [14]. BCL2L1 DNA copy number is increased in colorectal cancers compared to adenomas [15]. Myeloid cell leukemia sequence 1 (MCL1), is located in 1q21.2. MCL1 functions as an anti-apoptotic molecule and is capable of blocking apoptosis induced by various apoptotic stimuli, including etoposide, staurosporine, UV irradiation, calcium ionophore A23187, and c-Myc overexpression [16]. High levels of MCL1 expression were found in many different cancer types [10, 17]. MCL1 copy number gain is a frequent event in several cancers, like mantle cell lymphoma, lung cancer, and breast cancer [18, 19]. What is more, overexpression of MCL1 has also been reported to be correlated with poor survival and resistance to chemotherapeutic agents [10].

Above all, we can hypothesize that copy-number variations (CNVs) in BCL2L1 and MCL1 may be associated with cancer prognosis. To date and to the best of our knowledge, there have been rare studies addressing the roles of CNVs of BCL2L1 and MCL1 in NSCLC outcomes. In this study, we detected BCL2L1, MCL1 CNVs in DNA from NSCLC tumors tissues in a southern Chinese population; additionally, we conducted survival analyses to analyze prognostic values of BCL2L1, MCL1 CNVs on overall survival (OS).

Materials and Methods

Ethics statement

The study was approved by the institutional review boards of Tongji Medical College. All patients were informed about the aims of specimen collection and gave signed written consent in accordance with the ethical guidelines of Tongji Medical College.

Study subjects

All the patients were histopathologically confirmed, without preoperative chemotherapy or radiotherapy, and underwent lung cancer surgery in the Affiliated Tongji Hospital of Huazhong University of Science and Technology from October 2006 to June 2012. Only patients with fully characterized somatic CNV status and intact survival information were included in this study, and there was no selection bias (Table S1). Eventually, 516 patients were recruited, among which 390 patients provided formalin-fixed paraffin-embedded (FFPE) tissues; the other 126 tumor specimens were obtained during surgery, then snap frozen in liquid nitrogen, and finally stored at −80°C until usage. The tumor specimens selected for DNA isolation were verified to consist of a minimum of 80% tumor cells in each case on hematoxylin and eosin-stained tissue sections. All tumor specimens were histologically reviewed.

Data were collected on demographic characteristics, smoking status, alcohol intake, and family history of cancer. The definition of “ever smokers”, “never smokers”, “ever drinkers” and “never drinkers” could be found in Appended Method. Age at cancer diagnosis and additional clinical characteristics were extracted from medical records; TNM stages were defined according to American Joint Committee on Cancer Staging Manual, 7th edition [20].

Follow-up was designed to carry out every 3 months for the first year; every 6 months for the next 2 years, and every 12 months from the fourth year after surgery. Information on event (death) and event time was obtained by trained nurses and medical students via telephone interview. For this study, the last follow-up was performed on 31 July 2014.

DNA extraction and quality control

Genomic DNA was isolated from frozen and FFPE samples using the TIANamp Genomic DNA Kit DP304 (Tiangen,
Beijing, China) and TIANamp FFPE DNA Kit DP331 (Tiangen, Beijing, China), respectively.

PCR-based approaches to analyze genes copy-number changes in FFPE tissues have been successfully conducted [21, 22]. As suggested [21, 22], only high-quality DNA template was accepted: (1) with an OD260/280 ratio range of 1.7–1.9, as measured by the NanoDrop 1000 instrument (NanoDrop Technologies, Wilmington, DE); (2) without DNA degradation (tested by Gel electrophoresis, Fig. S1). All DNA working solutions were diluted to 5 ng/μL for final storage and usage.

**Primer design**

By searching the DGV database (http://projects.tcag.ca/variation/?source=hg19), we found the landmark of BCL2L1 CNV (chr20:30,252,261 .. 30,310,656), and then DNA sequence was downloaded from NCBI (http://www.ncbi.nlm.nih.gov/). Primer premier v5.0 software (Applied Biosystems, Foster City, CA, USA) was used to design primers for BCL2L1. The primers used for MCL1 [19] were previously described.

Single-copy genes RPPH1 and β-globin were used as reference genes. The primers for RPPH1 were also designed by Primer premier, and the primers for β-globin were extracted from published papers [23–25].

All primer sequences used in this study were available in Table S2. We subsequently conducted BLAST search (http://www.blast.ncbi.nlm.nih.gov) and UCSC In-Silico PCR (http://genome.ucsc.edu) to evaluate and confirm the specificity of these primers.

**Real-Time Quantitative Polymerase Chain Reaction**

BCL2L1 and MCL1 CNVs were tested by real-time quantitative polymerase chain reaction (RT-qPCR) relative to two sing-copy reference genes (RPPH1 and β-globin). To screen the CNV, combined use of two reference genes was suggested to produce a robust, reliable, and accurate quantification [26–28].

All PCRs were done on a 7900HT Sequence Detection System (Applied Biosystems) with Toyobo Thunderbird SYBR® qPCR Mix. The PCR reaction mixture (10 μL) contained 5 μL 2×SYBR mix, 3 μmol/L each of the forward and reverse primers, and 3 μL (5 ng/μL) template DNA. All PCR reaction mixes were prepared in bulk with 10% extra volume, allowing excess for pipetting waste and were transferred to the 384-well plate with multichannel pipette. The PCR conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 30 sec, and 72°C for 45 sec. Melting (dissociation) curve analysis was performed on every run to verify specificity and identify the PCR products. Test for BCL2L1, MCL1, RPPH1, and β-globin were done in triplicate in the same 384-well plate. The threshold cycle number (Ct) values were automatically determined by the ABI system.

Samples were successfully genotyped when Ct values for the triplicate fell within 0.3 units of each other; otherwise, the sample was retested. Triplicate wells of simplex (BCL2L1, MCL1, RPPH1 or β-globin) reactions containing a series of twofold dilutions of pooled germline DNA samples (30–1.0625 ng/reaction) from 50 randomly selected healthy individuals were used to determine the PCR efficiencies of each assay. Each plate also contained three negative controls (water) and calibrator DNA (this calibrator was verified to be without variations in these tested four genes by a whole genome copy-number array in China). The r² correlation for each standard curve was ≥0.98.

**Statistical analyses**

OS was the primary outcome measure which was calculated from the date of operation to the date of death from any cause or the date of last follow-up. Relative quantification of BCL2L1, MCL1 was performed by the 2−ΔΔCt method [29] with little modifications as described by Lazar et al. [26], and the equation is shown in **Appendix Method**. Consequently, BCL2L1 or MCL1 CNVs were dichotomized into two groups: “non-amplification” and “amplification”. 2−ΔΔCt value >1 was interpreted as “amplification”. Multivariate Cox proportional hazards regression analysis was used to evaluate the effect of CNV status and clinicopathological variables on OS, illustrated as hazard ratio (HR) with their corresponding 95% confidence interval (95% confidence interval [CI]). The Kaplan–Meier method was used to compare OS among different CNV status. Statistical analyses were conducted with SAS software (version 9.1.3; SAS Institute, Cary, NC). Statistical significance of the improvement in area under curve (AUC) after adding an explanatory factor was calculated by the Delong’s test [30]. All statistical tests were two-sided with a significance level of α = 0.05.

**Bioinformatics analysis**

CNV data and corresponding normalized gene expression data from tumor tissues for lung adenocarcinoma (LUAD) and Lung squamous cell carcinoma (LUSC) were obtained from The Cancer Genome Atlas (TCGA) data portal (http://cancergenome.nih.gov/, November 2014). This study meets the publication guidelines proved by TCGA (http://cancergenome.nih.gov/publications). The level 3 CNV segmentation data were retrieved and
processed using TCGA-assembler [31]. If the CNV value was higher than 0.2, we defined it as “amplification”; otherwise, “non-amplification”, as suggested by others scholars [32]. Differences in gene expression levels between CNV status were assessed by a Wilcoxon signed-rank test.

Table 1. Associations of patient demographic and tumor-related characters with OS.

| Parameter                      | Patients | Death | MST (months) | Univariate analysis¹ | Multivariate analysis² |
|--------------------------------|----------|-------|--------------|-----------------------|------------------------|
|                                | No.      | No.   |              | HR (95% CI)           | P                      |
| Age (years)                    |          |       |              |                       |                        |
| ≤58                            | 259      | 111   | 65.5         | 1.00                  | 1.00                   |
| >58                            | 257      | 123   | 57.6         | 1.16 (0.9–1.50)       | 0.254                  |
| Sex                            |          |       |              |                       |                        |
| Male                           | 389      | 173   | 63.3         | 1.00                  | 1.00                   |
| Female                         | 127      | 61    | 46.7         | 1.07 (0.80–1.43)      | 0.657                  |
| Smoking status                 |          |       |              |                       |                        |
| Never                          | 158      | 76    | 46.5         | 1.00                  | 1.00                   |
| Ever                           | 358      | 158   | 63.9         | 0.91 (0.69–1.20)      | 0.494                  |
| Alcohol intake                 |          |       |              |                       |                        |
| Never                          | 311      | 139   | 61.6         | 1.00                  | 1.00                   |
| Ever                           | 205      | 95    | 57.7         | 0.98 (0.75–1.27)      | 0.871                  |
| Family history of cancer       |          |       |              |                       |                        |
| No                             | 440      | 202   | 59.1         | 1.00                  | 1.00                   |
| Yes                            | 76       | 32    | NA           | 0.89 (0.61–1.29)      | 0.538                  |
| Histological types             |          |       |              |                       |                        |
| Squamous Carcinoma             | 242      | 106   | 67.7         | 1.00                  | 1.00                   |
| Adenocarcinoma                 | 249      | 124   | 46.5         | 1.22 (0.94–1.58)      | 0.137                  |
| Others                         | 25       | 4     | NA           | 0.29 (0.11–0.79)      | 0.015                  |
| TNM stage                      |          |       |              |                       |                        |
| Ia                             | 38       | 5     | NA           | 1.00                  | 1.00                   |
| Iib                            | 87       | 23    | NA           | 2.34 (0.89–6.16)      | 0.085                  |
| IIa                            | 62       | 24    | NA           | 3.78 (1.44–9.92)      | 0.007                  |
| IIb                            | 63       | 26    | NA           | 4.70 (1.80–12.24)     | 0.002                  |
| Illa                           | 168      | 102   | 23.3         | 8.35 (3.40–20.53)     | <.0001                 |
| IIIb                           | 28       | 21    | 16           | 12.64 (4.75–33.64)    | <.0001                 |
| IVa                            | 39       | 26    | 17           | 10.74 (4.12–28.05)    | <.0001                 |
| Missing                        | 31       |       |              |                       |                        |
| Laterality                     |          |       |              |                       |                        |
| Left                           | 236      | 117   | 41.9         | 1.00                  | 1.00                   |
| Right                          | 275      | 113   | 66.3         | 0.79 (0.61–1.03)      | 0.077                  |
| Others                         | 5        |       |              |                       | 0.93 (0.71–1.22)       | 0.589                  |
| Surgical approach              |          |       |              |                       |                        |
| Lobectomy or sublobectomy      | 442      | 181   | 85.3         | 1.00                  | 1.00                   |
| Pneumonectomy                  | 74       | 53    | 18.6         | 2.56 (1.88–3.49)      | <.0001                 |
| Chemotherapy³                  |          |       |              |                       |                        |
| No                             | 209      | 87    | NA           | 1.00                  | 1.00                   |
| Yes                            | 307      | 147   | 48.9         | 1.07 (0.82–1.40)      | 0.613                  |
| Radiotherapy³                  |          |       |              |                       |                        |
| No                             | 372      | 156   | 84.8         | 1.00                  | 1.00                   |
| Yes                            | 144      | 78    | 38.7         | 1.24 (0.95–1.63)      | 0.120                  |
| DNA source                     |          |       |              |                       |                        |
| Fresh                          | 126      | 33    | NA           | 1.00                  | 1.00                   |
| FFPE                           | 390      | 201   | 57.6         | 1.34 (0.92–1.95)      | 0.132                  |

FFPE, formalin-fixed paraffin-embedded; MST, median survival time; OS, overall survival; HR, hazards ratio; 95% confidence interval, 95% CI; not available, NA.

¹Univariate analysis.
²Adjusted by other variables in Table 1.
³Chemotherapy or Radiotherapy after operation.
Results

Basic characteristics and genotyping data

The demographic and clinical variables of 516 patients with NSCLC are shown in Table 1. The patients were aged between 22 and 80 years at diagnosis with a mean of 58.01 years and standard deviation of 9.56 years. There were more men than women (389 vs. 127) and more ever smokers than non-smokers (358 vs. 158); 250 of the patients had early-stage lung cancer (stages Ia, Ib, IIa, and IIb). A total of 242 patients had lung squamous carcinoma, and 249 had lung adenocarcinoma. The median follow-up time (MFT) for overall patients was 28 months, during when 234 (45.3%) patients died.

In multivariable analysis, TNM stage and surgical approach were found to be independent prognostic factors for NSCLC patients’ OS.

CNV of BCL2L1, MCL1, and OS

In this study, 64 (12.4%), 173 (33.5%) of 516 NSCLC patients were identified to carry amplifications of BCL2L1 and MCL1, respectively. Patients with amplifications of BCL2L1 exhibited significantly increased hazards of death, compared with those without amplifications (adjusted HR = 1.62, 95% CI = 1.10–2.40, P = 0.015, Table 2). For MCL1 CNVs, amplifications showed a strong association with shorter OS (amplification vs. non-amplification, adjusted HR = 1.39, 95% CI = 1.05–1.84, P = 0.020). For illustrative purpose, Kaplan–Meier curves of the associations with OS and CNV status are shown in Figure 1.

Different histological subtypes (LAUD and LUSC) have equal distribution of amplifications of BCL2L1 (chi-squared test, P = 0.910) and MCL1 (chi-squared test, P = 0.110). Subgroup analyses stratified by different histological subtypes showed that the prognostic effects of BCL2L1 and MCL1 failed to reach significance in the both groups of LAUD and LUSC (Table 2).

Bioinformatics analysis

To explore the effect of CNVs in MCL1 and BCL2L1 on the corresponding genes expressions, we derived CNV data (in level 3) for tumor tissues from 491 LAUD and 487 LUSC patients in September, 2014., and normalized gene expression data (in level 3) from 1084 LAUD and 1033 LUSC tissues in TCGA database (performed on 17 November 2014). Overall, there were 986 NSCLC patients had data on both CNV and gene expression. As shown in Figure 2, the amplification of

Table 2. The Associations between CNVs of BCL2L1, MCL1, and NSCLC OS.

| CNVs          | Number of Patients | MST (months) | Univariate analysis¹ | Multivariate analysis² |
|---------------|--------------------|--------------|-----------------------|------------------------|
|               | Death              |              | HR (95% CI)           | P          | HR (95% CI)           | P          |
| **BCL2L1**    |                    |              |                       |            |                       |            |
| Overall       | 452                | 200          | 65.3                  | 1.00       | 1.00                   | 1.00       |
| Non-amplification | 342            | 140          | 66.5                  | 1.00       | 1.00                   | 1.00       |
| Amplification  | 110                |              |                       |            |                       |            |
| Squamous carcinoma |              |              |                       |            |                       |            |
| Non-amplification | 214            | 89           | NA                    |            |                       |            |
| Amplification  | 28                 |              | 20.1                  | 1.88 (1.12–3.17) | 0.018  | 1.35 (0.76–2.39) | 0.307    |
| Adenocarcinoma |                    |              |                       |            |                       |            |
| Non-amplification | 221            | 108          | 45.3                  | 1.36 (0.81–2.30) | 0.251  | 1.62 (0.91–2.88) | 0.102    |
| Amplification  | 28                 |              | 37.6                  |            |                       |            |
| **MCL1**      |                    |              |                       |            |                       |            |
| Overall       | 343                | 140          | 66.5                  | 1.00       | 1.00                   | 1.00       |
| Non-amplification | 170            | 69           | NA                    |            |                       |            |
| Amplification  | 72                 |              | 36.7                  | 1.27 (0.85–1.89) | 0.249  | 1.17 (0.77–1.80) | 0.458    |
| Squamous carcinoma |              |              |                       |            |                       |            |
| Non-amplification | 158            | 68           | 57.2                  |            |                       |            |
| Amplification  | 91                 |              | 38.0                  | 1.31 (0.92–1.87) | 0.139  | 1.40 (0.93–2.09) | 0.105    |

CNVs, Copy-number variation; MST, median survival time; OS, overall survival; HR, hazards ratio; 95% CI, 95% confidence interval.
¹univariate analysis.
²Adjusted by age, gender, smoking status, alcohol intake, family history of cancer, histological types, TNM stages, laterality, surgical approach, chemotherapy, radiotherapy, and DNA source.
MCL1 was shown to be associated with higher expression levels of MCL1 mRNA (P = 0.005), compared with non-amplification. Nevertheless, no significant correlation was found between BCL2L1 CNV and mRNA expression levels (P = 0.382).

Receiver operating characteristic curve

The capacity of CNVs for the classification of OS in NSCLC patients was evaluated by the multivariate logistic regression model and receiver operating characteristic curve. As shown in Figure 3, with MCL1 CNVs, the AUC for predicted model was significantly improved to 74.1%, which is higher than the 72.2% AUC for the model only included age, sex and the independent prognostic variables (TNM stage and surgical approach) (P = 0.042, DeLong’s test). But there is no similar finding for the BCL2L1 CNV status (72.2% vs. 72.5%, P = 0.451, DeLong’s test).

Discussion

In this study, we found that amplifications of MCL1 and BCL2L1 were likely to modulate OS of NSCLC patients in a southern Chinese population. However, only MCL1 CNVs were found to be capable of influencing corresponding mRNA expression levels. The observed improvement of discrimination of NSCLC OS by MCL1 CNV status supports the prognostic impact of associations and potential clinical applications.

CNV is proposed to influence gene expression, possibly by altering gene dosage, disrupting coding sequences, or perturbing long-range gene regulation [33]. Meanwhile, a study found that 53% of the expression probes associated with a comparative genomic hybridization (CGH) clone, were located outside the CNVs that encompass the specific clone [34]. This suggested that gene expressions might be modulated by CNVs which were long-range apart. Therefore, it is biologically possible that BCL2L1 CNVs were not associated with corresponding mRNA expression levels.

MCL1 CNV was found to influence mRNA expression levels of MCL1. MCL1 can bind and sequester the pro-apoptotic proteins, Bax and Bak, and consequently suppress the release of cytochrome c from mitochondria into the cytoplasm [35]. In cytoplasm, cytochrome c could induce the activation of caspases and then launch macromolecular
degradation, which are the classic steps during mitochondrial pathway (intrinsic pathway) and apoptosis [36]. MCL1 plays a key role in cell immortalization, malignant transformation, and chemoresistance [16]. Silencing the expression of MCL1 with small interfering RNA (siRNA) potently killed a sub-group of NSCLC cell lines [17]. Over expression of MCL1 protein was also found in a subset of human NSCLC cells. And high level of MCL1 may protect lung cancer cells from death induced by a variety of pro-apoptotic stimuli [37]. MCL1 was suggested to be a critical molecule for chemoresistance in A549 cells associated with TGF-β-induced EMT [38]. The expression of MCL1 mRNA or protein has been associated with tumor progression and adverse patient outcome in multiple cancer types [39–43]. BCL2 inhibitors could induce apoptosis; this has been explored as a therapeutic approach in NSCLC [44]. A matter of interest is that, studies have suggested that MCL-1 expression could be related to resistance to BCL2 inhibitors (such as ABT-737) in lung cancer cell lines [45, 46]. Therefore, MCL1 CNV status might be considered for clinical decision of treatment using apoptotic inhibitor correlated with the Bcl-2 proteins.

We are aware that this study has some limitations. First, relatively small sample size and short median follow-up time were the major concern. However, by conducting power and sample size calculation [47], our study assessed MCL1 CNVs in 516 NSCLC patients, achieving a power of 0.964 to detect the potentially clinical significant differences in OS. Second, patients in this study may have received a wide variety of therapies, often sequentially or simultaneously. Only information about whether patients received postoperative chemotherapy/radiation therapy was available. In this study, chemotherapy and radiation therapy did not independently affect NSCLC OS and there were no significant differences among results of subgroups that accepted variant chemotherapy and radiation therapy (data not shown). Third, the prognosis predicting model was only built in a Southern Chinese population; the application and interpretation of our findings should be cautious and still needs further investigation. Fourth, lacking of information on resection margin status (R0 or R1) was another limitation. Finally, we acknowledge that this study will be more clinically critical if the association between the gene status and NSCLC-specific death are explored, on which we continue to work.

Our findings suggested that the amplifications of MCL1 might be associated with unfavorable NSCLC OS. However, the functional consequences of MCL1 amplifications and external validations need to be extensively investigated in the future.

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Conflicts of Interest
The author(s) indicated no potential conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. DNA degradation tested by Gel electrophoresis

Table S1. Characteristics of overall patients were recruited and patients were used in the current study

Table S2. Primer Sequence