Introduction

“Reactive oxygen species” (ROS) is a general term assigned to the chemical species that are formed upon the incomplete reduction of molecular oxygen, which include the superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (HO•). They are constantly produced by enzymatic or non-enzymatic metabolism in cells. Low levels of ROS can reversibly oxidize protein thiol groups and thereby act as cellular signaling messengers, while higher levels of ROS may damage fundamental cell processes by nonspecifically attacking proteins, lipids, and DNA [1]. To address the oxidative stress elicited by ROS, mammalian cells have developed an antioxidant defense system, which consists of antioxidant enzymes, as well as several non-enzymatic antioxidants such as glutathione (GSH), cysteine, thioredoxin (Thx) and vitamins [2,3].

Glutathione S-transferase (GSTs) compose a family of phase II detoxification enzymes that catalyze the conjugation of GSH to a wide range of endogenous and exogenous electrophilic compounds [4]. GSTs can be divided into three main categories: the cytosolic, mitochondrial and membrane-bound microsomal GSTs [5,6]. Among them, cytosolic GSTs exhibit genetic polymorphisms related to the susceptibility to carcinogenesis [5,7]. In mammals, seven classes of cytosolic GSTs have been recognized, namely, alpha (α), mu (μ), pi (π), sigma (σ), theta (θ), zeta (ζ) and omega (ω) [8,9]. GSTM1, which belongs to the GST mu class (GSTM1-5), is expressed at high levels in normal liver tissues [5]. The association between GSTM1 polymorphisms and cancer risks has been widely studied in multiple cancers and shows that the GSTM1 null phenotype may increase the risk of liver cancer, gastric cancer, breast cancer and prostate cancer [9-13]; however, the association was not significant in some meta-analyses in other cancers [14-17]. Moreover, the association of the GSTM1 null genotype with the outcome of cancer therapy has also been reported in previous studies in which controversial results were obtained [18-20]. The possible cause of this striking discordance may lie in the complexity of the antioxidant, which can either protected against cancer or increased cancer risk in different experimental and preclinical studies [21-25].

Despite the abundant evidence linking GSTM1 to cancer development, it remains unclear how GSTM1 affects the biological behavior of cancer cell in hepatocellular carcinoma (HCC) and which mechanism are implicated. HCC is one of the major malignant diseases and the third most common cause of death from cancer worldwide [26]. Oxidative stress has been investigated for many years as a possible cause of chronic liver injury and cancer development. Clarifying the underlying mechanism of GSTM1 in HCC may help improve the antioxidant-based therapeutic strategies in the future.

Materials and Methods

Tissue specimens

Forty-seven cancerous and adjacent noncancerous specimens were obtained from patients who provided informed consent and who underwent surgery for primary HCC at the Eastern Hepatobiliary Surgery Hospital (Shanghai, China) between 2010 and 2013. The corresponding non-tumor tissues were obtained at least 5 cm away from the primary tumor. The study was approved by the Committees for the Ethical Review of Research Involving Human Subjects of Second Military Medical University. Twenty-three had primary HCC for the Ethical Review of Research Involving Human Subjects of the 117th Hospital of the Chinese Liberation Army (PLA), Hangzhou, Zhejiang 310013, China. SMMC-7721 (p53 null wildtype).

Conclusion: GSTM1 downregulation may partially account for ROS-mediated oxidative damage and HCC carcinogenesis, and GSTM1 also regulate tumor progression by disrupting the ROS-P53 axis in HCC cells with different genetic background.
Gene expression analysis

Total RNA was extracted with TRIzol (Invitrogen). For RT-qPCR, 1 μg total RNA was reverse transcribed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, USA). Real-time PCR was performed using FastStart Universal SYBR Green Master (RoX) on the Applied Biosystems 7500 Sequence Detection system (Applied Biosystems, USA). Primer sequences are listed in Table S1.

Western blot analysis

The tissues and cells were lysed in RIPA lysis buffer (Beyotime, China). Identical quantities of proteins were separated using 10 %-15 % odium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, 0.45 μm, USA). After incubation with antibodies specific for GSTM1 (Abcam, ab113432), P53 (CST, #9282), Apoptosis Antibody Sampler Kit (CST, #9915), and Actin (Protein Tech, 60008-1), the blots were incubated with horseradish peroxidase (HRP)-coupled anti-mouse (CST, #7076) or anti-rabbit secondary antibodies (CST, #7074) and then detected with enhanced chemiluminescence (Fierce Biotechnology, USA). The β-actin protein was used as a loading control.

Plasmid construction and siRNA

The cDNA encoding GSTM1, P53 (wild type) or P53 (R249S) was PCR amplified by Taq polymerase (SinoBio) and subcloned into the TaqBamHI/Xhol site of pcDNA3.1 (+). GSTM1 siRNA or a nonspecific sequence were purchased from GenePharma (Shanghai, China).

Cell line culture and treatment

The cell lines SMMC-7721 (p53 wild type), MHCC-97H (p53 p.R249S), MHCC-97L (p53 p.R249S) and Hep3B (p53 null) were purchased from the cell bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China). All the cells were cultured in DMEM (High Glucose) (HyClone, USA) which contains 10 % fetal bovine serum (HyClone, USA) at 37 °C in 5 % CO₂, and subcultured every 2-3 days. The cells were seeded on six-well plates at a density of 3 × 10⁵/well or twelve-well plates at a density of 1 × 10⁵/well. The transfection was performed using the FuGene HD Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer’s instruction. The transfected cells were harvested at 48-72 h. For apoptosis analysis, cells were transfected with plasmid or siRNA for 24 h before the addition of doxorubicin (DOX, 1 μM for SMMC-7721, 2.5 μM for MHCC-97H/L or Hep3B), after 48-72 h, cells were harvested for tests.

Cell apoptosis and cell cycle distribution analysis

For FACS analysis of apoptosis and the cell cycle, adherent and floating cells were harvested, following procedures conducted according to the introduction of the KeyGEN cell apoptosis detection kit (KGa107) and the KeyGEN cell cycle detection kit (KGa511-KGa512).

Migration assays

For transwell migration assays, cells (0.5 – 4 × 10⁵) were seeded in 1 % PBS on 24 - well PET inserts (0.8 μm pore size, Millipore), while the lower chamber was filled with 700 μL 20 % serum medium. After 24 h, cells that passed through the filter were fixed in 4 % formaldehyde, stained with 0.05 % crystal violet and counted.

Vector construction and lentivirus production and the construction of stable cell line with GSTM1 overexpression

For the construction of lentiviral vector expressing the GSTM1 gene, GSTM1 cDNA was PCR-amplified and subcloned into the lentiviral vector GV260 Ubi-MCS-Luc-IRES-Puromycin using the In-Fusion™ PCR Cloning Kit (Clontech, USA). To produce lentivirus containing the GSTM1 gene, 293T cells were co-transfected with the resulting vector described above, pHelper 1.0, and pHelper 2.0 using Lipofectamine 2000 according to the manufacturer’s guidelines. SMMC-7721 and MHCC-97H cells were transfected with 2 × 10⁶ transducing units of GSTM1 overexpressing lentiviruses and were selected with 2 g/mL puromycin for two weeks. The stably expressed cell lines were identified using real-time PCR and WB.

In vivo tumorigenicity and metastasis assays

For xenografts, the stable cell line of SMMC-7721 cells (1 × 10⁶ NC or GSTM1 overexpressed) were suspended in 100 μL PBS and injected into the lower flank of a 4-6-week-old nude mice, and NC-transfected SMMC-7721 cells were injected into the opposite lower flank of the same nude mouse. After 3-4 weeks, the tumors were removed from the nude mice and photographed. Tumor diameters are measured with calipers, and the tumor volume in mm³ is calculated by the following formula: Volume = (width)² × length/2, the tumors were also weighted with an electronic balance. A tail vein injection model was used for lung colonization assays. The stable cell lines of MHCC-97H cells (2 × 10⁵ NC or GSTM1 overexpressed cells) were suspended in 100 μL PBS and injected into the mouse tail vein, the metastasis was monitored using the IVIS® Lumina system (Caliper Life Science, Hopkinton, MA) 10 min after the tail vein injection of 4.0 mg of luciferin (Gold Biotech, USA) in 50 μL of saline.

ROS assay

After transfected with the GSTM1 or pcDNA3.1 plasmid for 48 h, Wash cells once in 1X Buffer, DMEM medium containing 10 μM DCFH-DA (Nanjing Jiansheng Bioengineering Institute, China) was used to culture the cells for 30-60 min at 37°C, then, cells were harvested to the detect the fluorescence signal by flow cytometry at Ex/Em:485/535 nm.

GSH assay

The transfected cells were harvest at 48 - 72 h, then lysed with RIPA lysis buffer. Cell lysates were collected and used in the GSH assay kit (A006-2 Assay, Nanjing Jiancheng Bioengineering Institute, China). Different concentrations of GSH standard included in the kit (0, 5, 10, 20, 50 and 100 μg/μl) were strained with Reagents 2 and 3 contained in the kit for 5 min at room temperature and the absorbance was measured at 405 nm using a microplate reader (BioTek Instruments, Inc., USA) to construct a standard curve. The cell lysates also stained with Reagents 2 and 3, and the GSH concentration was calculated according to the standard curve.

Bioinformatic and statistical analysis

The results were compared using Student’s t test and one-way ANOVA, and the data are expressed as the means and standard deviations of at least three independent experiments. For the analysis of the correlation between the expression levels of GSTM1 and clinical features, Pearson chi-square tests or Fisher exact tests were used. Pearson’s correlation coefficient is exploited to measure the relevance between the expression levels of GSTM1 and P53. All the p values were two-tailed and were obtained using GraphPad Prism 6. Results were considered statistically significant at p < 0.05.

Results

GSTM1 is downregulated in HCC and associated with the metastatic phenotypes

To determine the expression level of GSTM1 in HCC, qRT-PCR and Western Blot assays were performed to analyze Forty-seven paired HCC tissue samples. Using GAPDH as the control, the results showed that GSTM1 is significantly downregulated in 66.7 % (32/47) of cancer tissues compared with the matching adjacent normal tissues.
The average mRNA and protein expression level of GSTM1 in HCC were significantly lower than those observed in the adjacent tissue (p < 0.001) (Figure 1A and Figure 1B). Moreover, as revealed in Table 1, the expression levels of GSTM1 were significantly associated with portal vein tumor thrombus (PVTT) (p = 0.0365). However, the Kaplan–Meier survival analyses (Figure 1C) demonstrated that the mRNA expression level of GSTM1 was not associated with the HCC patients’ overall survival time (lower percentage of 25 % and upper percentage of 25 %, p = 0.127) (TCGA, n=361, http://www.oncolnc.org). In addition, we analyzed some cancer data in the Oncomine microarray database (https://www.oncomine.org), which showed that GSTM1 is also downregulated in breast cancer (p<0.001) [27], colorectal cancer (p<0.001) and prostate cancer (p<0.001) [28] (Figure S1A, B, C).

**GSTM1 regulates cancer cell metastasis and DOX induced apoptosis in HCC cell lines**

To further explore the biological effect of GSTM1 in HCC, in vitro assays were performed in MHCC-97H, MHCC-97L, SMMC-7721 and Hep3B cells. First, we did not observe any differences in cell cycle distribution after GSTM1 overexpression or knockdown (Figure S3A), and MTS cell proliferation assays confirmed that GSTM1 overexpression and depletion had no effect on cell proliferation (Figure S3B). Then, cell motility was monitored using transwell assays in SMMC-7721 (p53 WT), MHCC-97H/L (p53 p.R249S) and Hep3B (p53 null) human HCC cell lines. Interestingly, we observed both pro-metastatic and anti-metastatic effects after GSTM1 overexpression in different cells. Specifically, GSTM1 promoted cell metastasis in SMMC-7721 (p < 0.01) and inhibited cell metastasis in MHCC-97H (p < 0.001); while it had no effect in Hep3B cells (Figure 2). Moreover, we investigated the potential role of GSTM1 in cell apoptosis. Apoptosis was induced by doxorubicin (DOX) treatment and evaluated by flow cytometry. In a manner similar to the above data, we could find

**Table 1. The correlation between GSTM1 expression and clinical pathological characteristics.**

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Figure 1: GSTM1 was downregulated in HCC.
(A, B) qRT-PCR and WB results showed that GSTM1 is downregulated at the mRNA level and protein level in HCC compared with paired adjacent non-tumor tissues, ΔCt = Ct(GSTM1) - Ct(GAPDH), ***p<0.001. N: Normal, T: Tumor. (C) The Caner Genome Atlas project (TCGA) dataset was analyzed to reveal the effect of the GSTM1 mRNA expression level on the overall survival (OS) of patients with HCC (https://www.oncolnc.org), p=0.127. Low: the lower percentage of 25 % HCC patients, High: the upper percentage of 25 % HCC patients.

both pro-apoptotic and anti-apoptotic effects in different cell lines. In detail, the overexpression of GSTM1 inhibited the DOX-induced cells apoptosis in SMMC-7721 (p < 0.01) and promoted the cell apoptosis in MHCC-97H (p < 0.01), ablated GSTM1 expression increased the DOX-induced cell apoptosis in SMMC-7721 and the decreased DOX-induced cell apoptosis rate in MHCC-97L. No obvious effect was found in Hep3B cells after GSTM1 overexpression or knockdown (Figure 3A). Additionally, the expression of apoptosis regulators, such as c-Caspase 3, c-Caspase 9 and c-PARP, also confirmed the opposite changes in cell apoptosis in different cell lines (Figure 3B and C).

**GSTM1 regulates cancer progression** *in vivo*

To study the biological effect of GSTM1 *in vivo*, we constructed firefly luciferase-labeled stable cell lines, SMMC-7721 (p53, WT) and MHCC-97H (p53, p.R249S), in which GSTM1 or empty vectors were overexpressed. The mouse xenograft models of SMMC-7721 cells showed significant increases in tumor volume and weight after GSTM1 overexpression (Figure 4A, B, C). For MHCC-97H (p53, p.R249S) cells, an intravenous tail injection experiment was employed to explore the effect of GSTM1 on tumor metastasis. The results were obtained for lung metastasis 60 days after intravenous tail injection. As shown in Figure 4D, E, the overexpression of GSTM1 can significantly inhibit MHCC-97H lung metastasis, and Kaplan–Meier survival analyses showed that nude mice injected with overexpressed GSTM1 had longer survival time compared with a control stable cell line of MHCC-97H (p < 0.05). Thus, these findings demonstrate that the forced expression of GSTM1 performed dual functions in HCC.

**GSTM1 disrupts the ROS-p53 signaling axis**

GSTM1 is a phase II detoxification enzyme that catalyzes the conjugation reaction of GSH with various electrophilic compounds and contributes to the removal of ROS. The inactivation of GSTM1 has been reported to increase oxidative stress [29]. We, therefore, examined the effect of GSTM1 in regulating the levels of ROS and GSH in SMMC-7721 and MHCC-97H cells. As shown in Fig. 5A and B, GSTM1 significantly decreased the ROS level and increase the GSH concentration in both SMMC-7721 and MHCC-97H. Since the overexpression of GSTM1 results in a decreased intracellular ROS level in both cell lines and the
Figure 3: The dual effects of GSTM1 on HCC cancer cell apoptosis in vitro.
(A) Flow cytometry was used to determine the apoptotic rates in the different groups. The data are shown as the mean ± SD, Student’s t test, *p < 0.05. (B) The protein expression levels of cleaved caspase9, cleaved caspase3 and cleaved PARP were determined by Western Blot analysis after DOX treatment.
GSTM1 expression level was negatively correlated with P53 expression at the mRNA level in HCC patients (Figure 5C, TCGA, n = 361, r = -0.1609, p = 0.0022), we speculated that the controversial biological effect of GSTM1 on cancer metastasis or apoptosis may be caused by the genetic background of the above cell lines in the p53 genotype. To confirm this hypothesis, western blot assays were employed to examine P53 expression after the overexpression or depletion of GSTM1. They showed that P53 is significantly downregulated after overexpression of GSTM1 and upregulated after the downregulation of GSTM1 in SMMC-7721 and MHCC-97H/L (Figure 5D). Recently, several studies have identified that mutant P53 executes non-transcription activities and fails to induce apoptosis in cancer cells [30,31]. To confirm the differential effects of p53 (p.R249S) and p53 (WT), we transfected p53 (WT or p.R249S) or co-transfected p53 (WT or p.R249S mutant) and Gstm1 in Hep3B (p53 null) cells and investigated cell metastasis and apoptosis. As shown in Figure 5E, F, the overexpression of P53 could inhibit cell migration and increase DOX-induced cell apoptosis, while P53 (R249S) overexpression could promote cell migration and inhibit DOX-induced cell apoptosis. More importantly, GSTM1 co-transfection could block the pro-apoptotic or anti-apoptotic effects of P53 (WT) or P53 (R249S), respectively. Therefore, GSTM1 may regulate tumor progression by disrupting the ROS-P53 axis in HCC cells with different genetic backgrounds.

Discussion

The link between ROS and cellular transformation was first identified in 1991 [32], and increasing attention has been paid to uncovering the biological roles and underlying mechanism of ROS in cancer development. Although controversial, the prevailing notion has shown that low or moderate levels of ROS may contribute to cancer cell proliferation and survival, while high levels of ROS may induce cancer cell apoptosis [33,34]. Cancer cells are characterized by increased aerobic glycolysis (termed the Warburg effect) and high levels of ROS [34,35], and the ROS scavenging systems also increase to balance ROS-mediated oxidative damage and maintain ROS homeostasis in cancer cells [36]. Based on the above ideas, there has been great interest in the exploration of antioxidants to prevent cancer, but the effect of antioxidant in cancer therapy remains controversial.

In the present study, we showed that an endogenous antioxidant gene Gstm1, which is highly expressed in the liver, was downregulated in HCC tissue. Although association studies between Gstm1 polymorphisms and cancer risks have identified that the Gstm1 null phenotype may increase the risk of liver cancer, how GSTM1 is mechanistically implicated in the development and progression of HCC has not been studied previously. As a member of the family of phase II detoxification enzymes, the overexpression of GSTM1 can significantly reduce ROS levels in SMMC-7721 and MHCC-97H cells (Figure 5A). These observations were coordinated with previous idea that ROS-driven oxidant stress contributes to cancer initiation and progression. For example, a large epidemiological study showed that vitamin E can reduce the risk of liver cancer [37].

Then, we applied in vitro and in vivo models to explore the function of GSTM1 in regulating cancer phenotypic characteristics. Interestingly, different phenomena were exhibited for the three types of HCC cell lines used. For SMMC-7721 (p53, WT) cells, GSTM1 overexpression promoted cancer cell metastasis and inhibited DOX-induced apoptosis, while GSTM1 depletion inhibited metastasis and increased apoptotic rates (Figure 2A and Figure 3A), supporting the notion that excess ROS in cancer cells can bring oxidative stress to a high level that triggers cell death. So far, various drugs have been reported to increase the ROS level directly or indirectly to promote oxidative stress-induced cell death. Drugs that affect ROS metabolism such as NOV-002 [38] and sulphasalazine [39] directly target major antioxidant pathways [34], while drugs that increase ROS indirectly such as platinum coordination complexes and anthracyclines [40], are mainly used clinically. Among them, doxorubicin (DOX) is an FDA-approved medicine for the treatment of different types of cancer. It can induce the generation of ROS through a non-enzymatic pathway that utilizes iron and an enzymatic mechanism that involves the mitochondrial respiratory chain [39]. These data support that increasing ROS levels to induce cancer cell death is a valid method for the treatment of cancer.

Figure 5: GSTM1 disrupts the ROS-p53 signaling axis.

(A) The ROS level of SMMC-7721 and MHCC-97H after transfection with GSTM1-pcDNA3.1 or pcDNA3.1. (B) The concentration of reductive GSH of SMMC-7721 and MHCC-97H after transfection with GSTM1-pcDNA3.1 or pcDNA3.1. (C) The TCGA data showed that GSTM1 expression is negatively correlated with the P53 expression at the mRNA level in HCC patients (n=361, p=0.0022). (D) The protein expression level of P53 was downregulated by GSTM1 in SMMC-7721 and MHCC-97H/L. (E) The DOX induced cell apoptosis rate of Hep3B after transfected with GSTM1 or (and) P53 (WT or R249S). (F) The effect of P53 (WT or R249S) on Hep3B cell migration was determined using Millipore Transwell chambers.

However, we also observed a contradictory effect of GSTM1 in regulating cancer metastasis (Figure 2B) and apoptosis (Figure 3A) in p53 (p.R249S) cell lines, MHCC-97H and MHCC-97L, compared to SMMC-7721 cells. Interestingly, GSTM1 showed no regulatory roles in cell motility or apoptosis in the p53 null liver cell line, Hep3B. Based on the above analysis, we speculated that the ROS-P53 signaling axis may play a crucial role in defining the consequence of GSTM1 overexpression. Coordinately, we then found that GSTM1 inhibited the protein levels of P53 in all tested HCC cell lines. Our data was consistent with a previous study, revealing that exogenous antioxidants (NAC or Trolox) can reduce the amount of P53 [22]. More importantly, in Hep3B (p53 null) cells, we confirmed that mutant P53 (R249S) indeed plays an opposite role in regulating cell apoptosis compared to wild type P53. This strongly suggests that the controversial roles of GSTM1 in different cell lines may, at least in part, result from the status of p53. Recently, several in vitro and xenograft models have confirmed the ability of mutant P53 in driving cancer cell migration and growth via gain-of-function (GOF) and loss-of-function (LOF) [41,42]. Codon 249 mutation (c.747G>T; c.747G>C p.R249S), which is correlated with exposure to aflatoxin B1, is the greatest hotspot in liver tumors, accounting for 24.25 % (290/1196) (http://p53.iarc.fr) [43]. This mutant causes local structural changes, which can increase the flexibility of P53 and disrupt the DNA-binding surface [44,45]. Therefore, we suggest that in p53 (p.R249S) mutant cell lines, although the ablation of GSTM1 results in higher ROS levels, it fails to induce ROS-triggered apoptosis due to P53 malfunction.

In conclusion, our data indicates that GSTM1 downregulation may partially account for ROS-mediated oxidative damage and HCC carcinogenesis. Intriguingly, we also provide evidence that the depletion of GSTM1 produces a high level of ROS and induces apoptosis in HCC cells, while the mutation of P53 may confer controversial properties to cancer cells. These observations suggest that the status of P53 should be considered during antioxidant therapy for HCC or other cancers.
Conflicts of Interest
The authors declare that they have no competing interests in this section. All authors have reviewed the manuscript and approve it for publication. We claim that none of the materials in this paper has been published or is under consideration for publication elsewhere.

Acknowledgement
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Ethics Approval and Consent to Participate
The study was approved by the Committees for the Ethical Review of Research involving Human Subjects from the Second Military Medical University.

References


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Supplementary Materials

Figure S1: GSTM1 was downregulated in Breast carcinoma, colon Adenocarcinoma and prostate carcinoma.

(A, B, C) The mRNA expression level of GSTM1 was determined using the Oncomine microarray database (https://www.oncomine.org) in normal and cancer tissues. In the box plot, the boxes represent the interquartile range, the whiskers represent the 10th - 90th percentile range, and the bars represent the median, *** p<0.001.

Figure S2: Analysis the expression of GSTM1 after transfection of plasmids or siRNAs.

(A, B) qRT-PCR and western blot analysis for GSTM1 expression after transfected with plasmid pcDNA3.1 (or GSTM1) and siRNAs in SMMC-7721. (C) western blot analysis for GSTM1 expression after transfected with plasmid pcDNA3.1 (or GSTM1) and siRNAs in MHCC-97H/L.
Figure S3: GSTM1 had no effect on HCC cancer cell cycle distribution and cell proliferation in vitro.

(A) SMMC-7721, MHCC-97H/L and Hep3B cells were transfected with the GSTM1-pcDNA3.1 overexpression plasmid (or vector) or siRNAs. Flow cytometry was used to determine the distribution of cell cycle stages. (B) The proliferation of SMMC-7721 and MHCC-97H/L cells were measured by MTS experiments after being transfected with the GSTM1-pcDNA3.1 overexpression plasmid (or vector) or siRNAs.

Table S1: Primer sequences.

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