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3D culture system containing gellan gum restores oncogene dependence in ROS1 rearrangements non-small cell lung cancer

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ABSTRACT

The ROS1 fusion gene has been identified in approximately 1% of non-small cell lung cancer (NSCLC) cases. Several clinical studies have highlighted ROS1 as a promising therapeutic target because crizotinib, a multi-targeted drug against ROS1, ALK, and the MET proto-oncogene, has elicited remarkable responses in ROS1-rearrangements NSCLC. However, acquired resistance mediated by ROS1 kinase domain mutations has been identified and a system to assess ROS1 inhibitors for these resistant mutations is necessary for the promotion of drug development. Publicly available NSCLC cell lines harboring the ROS1 fusion gene are limited to only HCC78 cells carrying SLC34A2-ROS1. This cell line exhibits resistance to ROS1 inhibitors through activation of the EGFR pathway, although the cells were established from ROS1-TKI naïve pleural effusion. Here, we demonstrate that 3D culture with gellan gum can restore the ROS1 oncogene dependence of HCC78 cells by upregulating the expression of the ROS1 fusion gene and reducing the activity of the EGFR pathway. Moreover, we established the HCC78xe3 cell line, a subclone of the HCC78 cell line, by repeated *in vitro* cultures from tumor xenografts and created xenograft tumors three times using *in vitro* cultured cells. This eventually enabled us to engraft and stably grow the cells *in vivo*, and subsequently evaluate various ROS1 inhibitors against HCC78xe3 cells by overexpressing crizotinib-resistant mutations in the ROS1 kinase domain including G2032R and D2033 N. We newly found that lorlatinib, a next generation ROS1/ALK inhibitor, remain the activity against D2033 N mutation. Furthermore, we demonstrated that HCC78xe3 cells expressing SLC34A2-ROS1 G2032R, and D2033 N, but not wild type (WT) cells, were resistant to crizotinib *in vivo*. Taken together, our data suggested that 3D cultures of HCC78 might reflect the features in patients and this new system will be a useful tool for evaluating ROS1 inhibitors.

1. Introduction

Chromosomal rearrangements of receptor tyrosine kinase (RTK) have been identified as driver oncogenes in various cancers including non-small cell lung cancer (NSCLC) [1]. These gain-of-function genetic alterations in the tyrosine kinase coding gene constitutively activate both the kinase and its downstream pathway, resulting in cancer development [1]. ROS1 encodes orphan RTK proto-oncogenes related to the ALK/leukocyte tyrosine kinase and insulin receptor RTK families [2]. Chromosomal rearrangements of ROS1 in NSCLC were discovered in 2007 [3]. At present, 1%–2% of NSCLC patients have been observed to harbor ROS1 rearrangements [1]. Further sequencing analysis of NSCLC tumors elucidated the presence of several partner genes fused with ROS1, including CD74 (40%–50%), EZR (14%–15%), SLC34A2 (12%), and SDC4 (6%) [4].

ROS1 has been demonstrated to be a promising therapeutic target because pharmacologic inhibition of the gene showed remarkable clinical benefits [4]. Crizotinib, a multi-targeted drug against ROS1/ALK/MET, has gained approval in various countries including the United States as the first line treatment for ROS1 fusion-positive NSCLC patients. However, most patients developed resistance to crizotinib after the initial response and approximately 50%–60% of crizotinib-resistant tumors harbor mutations in the ROS1 kinase domain [4]. ROS1 G2032R mutation, located in the

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solvent front region, is the most common crizotinib-resistant mutation (approximately 40% of resistant biopsies) and interrupts drug binding through steric hindrance [4,5]. In addition, the existence of other resistant mutations has emerged, including D2033 N mutation in the solvent front region that results in ROS1 losing electrostatic interactions with crizotinib as well as S1986F mutation [6,7]. Clinical studies of next generation tyrosine kinase inhibitors (TKI) against ROS1 are ongoing, including lorlatinib, cabozantinib, entrectinib, brigatinib, ceritinib, DS-6051b, and TPX-0005, some of which also inhibit ALK because of its high homology with ROS1, specifically, 49% amino acid sequence identity in the kinase domain and 77% identity in the adenosine triphosphate (ATP) binding region [4,8]. Previous case reports have identified cabozantinib as a potent ROS1 inhibitor to overcome ROS1 D2033 N mutations in the clinical setting [6], and we previously reported that cabozantinib is active against G2032R *in vitro* [9].

To date, Ba/F3, a murine pro B cell line, transduced with the ROS1 fusion oncogene has mainly been used for investigating crizotinib-resistant mutations and evaluating the inhibitory efficacy of next generation ROS1 inhibitors because publicly available NSCLC cell lines harboring ROS1 rearrangements are limited. HCC78 is a publicly available cell line carrying the SLC34A2-ROS1 fusion gene, established from the pleural effusion of an NSCLC patient [3]. Unfortunately, HCC78 has been reported to lose ROS1 dependence because of the activation of the EGFR pathway [10]. Thus, the establishment of an assay system for assessing ROS1 inhibitors using NSCLC cells is needed.

Third dimension (3D) culture systems such as spheroid and organoid cultures that mimic tumor growth in tissue have been actively developed, and altered responses have often been observed [11,12]. Low-acyl gellan gum is one of the functional polymers that enable cells to be cultured in 3D without aggregation and sedimentation [13]. This 3D sphere culture system containing gellan gum has been demonstrated for large-scale human pluripotent stem cell production including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) [13].

In the current study, we determined that FCeM medium, an RPMI-1640 and Ham's F12 based medium containing gellan gum, restored the ROS1 dependence of HCC78 cells. Moreover, we established the HCC78xe3 cell line, a subclone of HCC78 cells capable of engrafting and growing *in vivo*. We subsequently evaluated the inhibitory potency of ROS1 inhibitors to HCC78xe3 cells overexpressing ROS1 WT, G2032R, and D2033 N mutations. We found that lorlatinib is potent against ROS1 D2033 N, and appears to be resistant to G2032R. Furthermore, we demonstrated that HCC78xe3 cells expressing SCL34A2-ROS1 G2032R and D2033 N tended to resistant to crizotinib *in vivo* in accordance with the results in the 3D culture condition. Overall, our results supported the use of HCC78 in 3D FCeM culture conditions for evaluating the activity of ROS1 inhibitors.

2. Materials and methods

2.1. Cell culture and reagents

HCC78 was obtained from DSMZ. HCC78xe3 cell is a subclone of HCC78, generated by repeating subcutaneously implantation and *in vitro* cell culture for 3 times. HCC78 and HCC78xe3 were maintained in culture dishes (IWAKI, 3020-100) in RPMI-1640/F-12 medium with 20 mM Hepes and 7.5% fetal bovine serum (FBS) (standard RP/F12 medium). HCC78xe3 cells expressed SLC34A2-ROS1 were sustained in ultra-low attached dishes (Corning, 3262) in ACL-4 medium containing 1% FBS. Ba/F3 cells that expressing CD74-ROS1 fusion gene were cultured in low-glucose DMEM containing 10% FBS. All media were supplemented with 100 units/ml penicillin, and 100 μg/ml streptomycin. The drugs used in the experiments and the companies from which they were purchased are indicated in Table S1.

2.2. Establishing Ba/F3 and HCC78xe3 cells overexpressing ROS1D2033 N fusion gene

Ba/F3 expressed CD74-ROS1 D2033 N as well as HCC78xe3 transduced SLC34A2-ROS1 D2033 N were established as previously described using the following primers: Fw: GGAACTGATGGAGGGAGGAAACCTTCTTACTTATTTGCG, Rv: CGCAAATAAGTAAGAAGGTTTCCCTCCCTCCATCAGTTCC [14].

2.3. Cell viability assay

Cell viability assay was performed as previously described [14]. For evaluating the growth inhibition of HCC78 to ROS1 inhibitors in 3D FCeM culture condition, the cells were pre-cultured for 3 days in FCeM medium containing 50% RPMI-1640/F-12, 50% FCeM-R (Nissan Chemical), 7.5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin, or in ACL-4 medium (invitrogen) supplemented 1% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in Ultra-low attachment culture dishes (Corning). Cell viability was evaluated by CellTiter-Glo Luminescent Cell Viability Assay (Promega).

2.4. Western blot analysis

Western blot analysis was performed as previously described [14]. Primary antibodies are shown in Table S2.

2.5. Quantitative RT-PCR analysis

Quantitative RT-PCR (qRT-PCR) was conducted by using Fast SYBR green Master Mix (Roche). The reaction was performed by the LightCycler 96 system (Roche). Primer design has been described in Table S3.

2.6. Animals and subcutaneous xenograft model

All mice studies were performed in line with the Institutional Animal Care and Use Committee-approved animal protocols according to the institutional guidelines.

2.5×10^6 cells were suspended in HBSS containing 50% matrigel matrix growth factor reduced (BD Biosciences) and subcutaneously implanted into the back of female BALB/c nude mice. Following the tumor volume reached 100−200 mm^3, Crizotinib (50 mg/kg) was orally administrated once a day for eight consecutive days. Tumor volume was calculated as length × $width^2$ × 0.5 (mm^3).

3. Results

3.1. 3D culture system containing gellan gum restores the ROS1 dependence of HCC78

HCC78 is one of the few publicly available NSCLC cell lines harboring ROS1 rearrangements, however, previous report has shown HCC78 was resistant to ROS1 inhibitors via activation of the EGFR pathway [10]. We confirmed that HCC78 cells were resistant to various ROS1 inhibitors, including crizotinib, lorlatinib, cabozantinib, entrectinib, ceritinib, and brigatinib. To assess the contributions of the EGFR pathway that induce resistance in HCC78 cells to ROS1 inhibitors, we performed combination treatment of crizotinib with and without a high dose of gefitinib. We found that the combination treatment reduced the IC_{50} (50% growth inhibitory constant) of crizotinib compared to

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