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Research paper

mTOR inhibitor temsirolimus and MEK1/2 inhibitor U0126 promote chromosomal instability and cell type-dependent phenotype changes of glioblastoma cells

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ABSTRACT

The phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and the RAF/mitogen-activated and extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways are frequently deregulated in cancer. Temsirolimus (TEM) and its primary active metabolite rapamycin allosterically block mTOR complex 1 substrate recruitment. The context-/experimental setup-dependent opposite effects of rapamycin on the multiple centrosome formation, aneuploidy, DNA damage/repair, proliferation, and invasion were reported. Similarly, the context-dependent either tumor-promoting or suppressing effects of RAF–MEK-ERK pathway and its inhibitors were demonstrated. Drug treatment-mediated stress may promote chromosomal instability (CIN), accelerating changes in the genomic landscape and phenotype diversity. Here, we characterized the genomic and phenotypic changes of U251 and T98G glioblastoma cell lines long-term treated with TEM or U0126, an inhibitor of MEK1/2. TEM significantly increased clonal and non-clonal chromosome aberrations. Both TEM and U0126 affected copy number alterations (CNAs) pattern. A proliferation rate of U251TEM and U251U0126 cells was lower and higher, respectively, than control cells. Colony formation efficiency of U251TEM significantly decreased, whereas U251U0126 did not change. U251TEM and U251U0126 cells decreased migration. In contrast, T98GTEM and T98GU0126 cells did not change proliferation, colony formation efficiency, and migration. Changes in the sensitivity of inhibitor-treated cells to the reduction of the glucose concentration were observed. Our results suggest that CIN and adaptive reprogramming of signal transduction pathways may be responsible for the cell type-dependent phenotype changes of long-term TEM- or U0126-treated tumor cells.

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1. Introduction

The phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and the RAF/mitogen-activated and extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways are the key signal transduction pathways responsible for integrating and decoding the different environmental signals. Both pathways are involved in regulation of all aspects of cell biology and frequently deregulated in cancer (McCubrey et al., 2007; Zoncu et al., 2011). There are multiple levels of interconnection with many points of convergence, cross-talk, and feedback loops between these pathways with a context-dependent ability to activate or inhibit each other (Aksamitiene et al., 2012; Mendoza et al., 2011).

MTOR serine/threonine kinase interacts with the regulatory proteins to form mTOR complex 1 (mTORC1), a highly integrated signaling node, which couples nutrients, growth factors, hormonal signals, and oxygen availability with the regulation of protein and lipid synthesis, glycolysis, pentose phosphate pathway, ribosome, mitochondrial, and lysosome biogenesis, autophagy, cellular

Abbreviations: aCGH, Array comparative genome hybridization; CIN, Chromosomal instability; CCAs, Clonal chromosome aberrations; CNAs, Copy number alterations; EVE, Everolimus; MEK, Mitogen-activated and extracellular signal-regulated kinase kinase; mTOR, Mammalian target of rapamycin; NCCAs, Non-clonal chromosome aberrations; TEM, Temsirolimus; TMZ, Temozolomide.

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senescence among other processes (Haissaguerre et al., 2014; Iadevaia et al., 2014; Puertollano, 2014; Shimobayashi and Hall, 2014; Xu et al., 2014). Temsirolimus (TEM) and everolimus (EVE) and their primary active metabolite rapamycin are the competitive inhibitors for the mTOR protein substrates, form a complex with FK506-binding proteins (FKBPs), then bind to mTOR and allosterically block mTORC1 substrate recruitment. However, some but not all of the functions of mTORC1 are impaired by rapamycin (Haissaguerre et al., 2014; Iadevaia et al., 2014; Puertollano, 2014; Shimobayashi and Hall, 2014; Xu et al., 2014). The cell type-dependent mTORC2 inhibition by rapamycin and the mTOR-independent rapamycin off-target effects are discussed below.

According to the National Library of Medicine's Hazardous Substances Data Bank (HSDB) (Fonger et al., 2014), TEM or rapamycin were not genotoxic/clastogenic in the *in vitro* bacterial and mammalian and *in vivo* mouse assays (*e.g.*, bacterial reverse mutation in *Salmonella typhimurium* and *Escherichia coli*, forward mutation in mouse lymphoma cells, chromosome aberrations in Chinese hamster ovary cells, and mouse micronucleus assays). In contrast, rapamycin caused supernumerary centrosomes, multipolar mitotic spindles, and aneuploidy in yeast and mammalian cells (*e.g.*, CHEF/18 cells and primary lymphoblastoid cell lines). Centrosome amplification was observed in CHEF/18 cells growing in the presence of rapamycin for just several generations (Bonatti et al., 1998, Bonatti et al., 2005). Furthermore, in yeast, rapamycin treatment repressed transcription-coupled DNA repair by competitively releasing factors interacting with Fpr1 (homolog of human FKBP12) that subsequently repress repair rather than due to inhibition of Tor activity (Limson and Sweder, 2010). On the other hand, rapamycin prevented the supernumerary centrosomes in *TSC1*(−/−) or *SP1*(−/−) mouse embryonic fibroblasts, which are characterized by an increased number of centrosomes and DNA content changes (Astrinidis et al., 2006, Astrinidis et al., 2010). Depending on an experimental setup, microencapsulated and enterically released rapamycin (eRapa) prevented (or did not) carcinogen-induced, inflammation-driven skin cancer. eRapa pre-treatment reduced DNA damage *in vitro* (phospho-H2AX staining for DNA double-strand breaks) and DNA damage and a cancer incidence *in vivo* in dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)-treated fibroblasts or skin without classical mTORC1 inhibition or effects on known proinflammatory mediators. However, eRapa cancer prevention and DNA damage reduction properties were abrogated when eRapa was given after DMBA-induced DNA damage (Dao et al., 2015).

The clarification of these and other outlined below controversial context-dependent observations is needed. Firstly, although eRapa extended mice lifespan by primary postponing lethal neoplastic disease (Ehninger et al., 2014), rapamycin is carcinogenic in rodents (promoted lymphoma, hepatocellular adenoma and carcinoma, and testicular adenoma), according to the National Library of Medicine's HSDB (Fonger et al., 2014). Secondly, the use of mTOR inhibitors is associated with higher risk of fatal adverse events in patients with advanced solid tumors (Choueiri et al., 2013; Qi et al., 2013; Sivendran et al., 2014). An increased relative risk of overall and severe metabolic adverse events and immunosuppression are the pronounced features of patients treated with mTOR inhibitors; however, adverse effects resulting from the possible genotoxic effects of mTOR inhibitors cannot be excluded. Thirdly, there are about twenty current clinical studies using mTOR inhibitors for the treatment of glioma (Pachow et al., 2015). Several phase II studies with recurrent glioblastoma have already reported no efficacy of TEM alone or in the combination with temozolomide, sorafenib, bevacizumab, or erlotinib (Pachow et al., 2015). Drug treatment-mediated stress may promote chromosomal instability (CIN), accelerate changes in the genomic landscape, tumor subclonal architecture and, eventually, favor cancer evolution (Duesberg et al., 2007; Liu et al., 2014; Stepanenko and Kavsan, 2012a; Stepanenko and Dmitrenko, 2015a; Stepanenko et al., 2015). A role of CIN in the mTOR inhibitor-based therapy failure was not addressed.

U0126 is an experimental MEK1/2 inhibitor with the well-characterized off-target effects (Stepanenko and Dmitrenko, 2015b). U0126 was never used in clinic due to its pharmacokinetic properties; however, it has been widely used *in vitro* and *in vivo* to elucidate the functions of MEK1/2 and their downstream targets ERK1/2 since the first report (Favata et al., 1998). Studies on *in vitro* and *in vivo* models demonstrated context-dependent either tumor-promoting or suppressing effects of RAF–MEK-ERK pathway and its inhibitors in cancer (Deschênes-Simard et al., 2014; Paraiso et al., 2014; Sanchez-Laorden et al., 2014; Stengel et al., 2008). Similarly, ERK activation in human cancers was linked to either good or bad prognosis (Deschênes-Simard et al., 2014). Generally, the antagonistic functional duality of cancer genes and the opposite context-dependent oncogenic/tumor suppressive effects of cancer genes and their inhibitors are widespread phenomena (Lou et al., 2014; Stepanenko et al., 2013).

Here, we characterized the genomic and phenotypic changes of U251 and T98G glioblastoma cell lines long-term treated with TEM or U0126 *in vitro*. We found that TEM significantly increased the number of clonal and non-clonal chromosomal aberrations (CCAs/NCCAs). Both TEM and U0126 affected copy number alterations (CNAs) pattern. The cell type-dependent changes in proliferation, colony formation efficiency in soft agar, migration, the sensitivity to the reduction of the glucose concentration, and drug cross-resistance were observed. Our results suggest that CIN and an individual pattern in the activation of signal transduction proteins may be responsible for the cell type-dependent phenotype changes of long-term TEM- or U0126-treated cells.

2. Materials and methods

2.1. Cell lines

Human glioblastoma U251 (received from the Bank of Cell Lines from Human and Animal Tissues, R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, Kyiv, Ukraine) and T98G (received from ATCC) cell lines were grown in DMEM (HyClone, Thermo Scientific, UK) supplemented with 10% FBS (HyClone, Thermo Scientific) and 100 μg/ml penicillin/100 u/ml streptomycin (Sigma, USA) in an environment of 95% air/5% CO_2.

2.2. Long-term treatment of glioblastoma cells with TEM and U0126 in vitro

100 mM stocks of TEM and U0126 (Abcam Biochemicals, USA) were prepared in 100% dimethylsulfoxide (DMSO), aliquoted and kept at −20 °C. U251 and T98G cell lines were seeded onto a panel of 10 cm culture dishes and treated in parallel with 5 μM TEM once a week for 5 weeks (U251TEM and T98GTEM) or 20 μM U0126 twice a week for 5 weeks (U251U0126(1), U251U0126(2), and T98GU0126), followed by two weeks of washout in the TEM/U0126-free medium before *in vitro* tests. DMSO did not exceed 0.1% by volume in culture plates.

2.3. Conventional cytogenetics

Chromosome samples were prepared as described previously (Stepanenko et al., 2015). Twenty metaphases were described for chromosome abnormalities, according to the International System for Human Cytogenetic Nomenclature 2013. Structural clonal chromosome aberrations (CCAs) were defined as aberrations found at least in two cells among 20 examined metaphases, whereas non-CCAs (NCCAs) as aberrations detected in only one cell. The frequency of structural NCCAs in cell line was calculated by dividing the

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