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RESEARCH ARTICLE

FASTING ENHANCES TKI EFFICACY ON THYROID CANCER CELLS.

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Abstract

Tyrosine kinase inhibitors (TKIs) have emerged as a novel cancer therapy for patients with poorly differentiated, anaplastic or medullary carcinomas unresponsive to conventional treatments. The association between fasting mimicking diets and TKIs has been explored with encouraging results showing a better response with less side effects due to TKI toxicity.
Here we report the role of fasting in reducing cell survival and in potentiating the anticancer activity of TKIs (Lenvatinib, Sorafenib, Vandetanib and Cabozatinib) in cultured medullary, follicular, papillary and anaplastic thyroid cancer cells. In particular, fast mimicking diet reduced cell growth in all cell types but potentiated the anticancer activity of TKIs only in follicular, medullary and TPC1 thyroid cells. TKIs significantly reduced ERK1-2 activation after 1 h in all cell types. Prolonged exposure to TKIs resulted in an improvement of ERK1-2 phosphorylation which was inhibited in the presence of starvation.
We demonstrated thyroid cancer cell susceptibility to fasting and validated its role in potentiating anticancer activity of TKIs by strengthening ERK1/2 signalling inhibition.

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Introduction:-

Differentiated thyroid carcinoma (DTC) includes the papillary and follicular histotype and their variants and accounts for more than 80% of all thyroid cancers. Undifferentiated (also called anaplastic) thyroid cancer (ATC) is still derived from the follicular epithelium but it is characterized by the almost complete loss of thyroid differentiation. Approximately 5–10% of patients with DTC develop distant metastases of whom two-thirds present with iodine refractory disease (1). Medullary thyroid carcinomas (MTCs) account for 5–8% of all thyroid cancers, and distant metastases that are present at diagnosis in approximately 20% of cases. These patients often suffer from a progressive disease despite local treatment such as radiofrequency ablation or external beam radiation therapy or systemic cytotoxic chemotherapies (2). In recent years, the therapeutic alternative that has proven to be the most effective in terms of progression free survival (PFS) in thyroid cancer refractory to conventional therapies is the use of thyrosin kinase inhibitors (TKIs) (3-5). Because of the effects of these drugs on the genetic pathways involved in thyroid oncogenesis such as MAPK, PI3K-AKT, their use is considered as a valid choice in advanced diseases (6). However, the use of these systemic drugs is not devoid by the onset of multiple significant side effects and are often linked to a resistance mechanism that can leads to a progression of the disease (7).

In literature, it has been reported that cancer cells are unable to adapt to starvation (8-11). Normal cells respond to starvation by inhibiting growth promoting signaling and protein synthesis. On the contrary, cancer cells amplify these processes with final activation of caspases and apoptosis due to oxidative stress (8-10). Thus, the idea that cancer could be treated with a combination of fasting cycles and chemotherapy. Recently, it has been reported that in several cancer cells, starvation condition increases the ability of commonly administered TKIs, to block cancer cell growth, to inhibit the MAPK signaling pathway and to strengthen E2F-dependent transcription inhibition (11).

Furthermore, in cancer xenografts models, both TKIs and cycles of fasting, slowed tumor growth and, when combined, these interventions were significantly more effective than either type of treatment alone (11). No similar studies exist, at the moment, on thyroid cancer refractory to conventional therapies.

Material and Methods:-

Reagents

Lenvatinib (E7080), Sorafenib, Vandetanib (ZD6474) and Cabozatinib (XL184, BMS-907351) were purchased from Selleckchem (Aurogene, Rome, Italy). Stock solutions were prepared according with the provided technical sheet. All media, sera and reagents for cell culture were purchased from Euroclone (Milan, Italy).

Cell lines

Hth7, 8505c, Cal62, C643 were a kind gift of Prof. Bellur S. Prabhakar (College of Medicine, Chicago, IL). TPC1 and BCPAP were kindly donated by Prof. M. Santoro (University of Naples Federico II, Italy). RO-82-W-1 and TT were purchased from Banca Biologica and Cell factory (San Martino, Italy). BCPAP, Hth7, C643, 8505c were cultured in DMEM high glucose; TPC1 and Cal62 were cultured in DMEM high glucose supplemented with 2mM L-Glutammine; TT and RO-82-W-1 were cultured in RPMI-1640 with 2mM L-Glutammine. Media were supplemented with penicillin/streptomycin and with 10% fetal calf serum (FCS).

Genetic analysis

Genomic DNA was extracted using the QIAamp DNA Micro Kit (Qiagen, Milan, Italy) following kit instructions. RNA was extracted using miRNeasy Mini Kit (Qiagen, Milan, Italy). DNA/RNA quality and concentrations were assessed by NanoDrop One (Thermo Scientific, Milan, Italy). 1 µg of RNA with rate values between 1.7 and 2 was reverse transcribed into complementary DNA (cDNA) using iScriptTM cDNA Synthesis Kit (Biorad, Milan, Italy) in a final volume of 50 µl. Point mutations of BRAF, RAS, RET and PAX8/PPARγ rearrangement were searched by end point PCR using specific primers (12-14). For point mutations, PCR products were analyzed with DHPLC and positive samples directly sequenced to confirm the presence of the mutation. For RET/PTC 1 rearrangement q-RT-PCR was used as described (15).

Viability assay

$1.5X10^3$ 8505c, TPC1, BCPAP, Hth7; $3X10^3$ C643, Cal62, TT and $2X10^3$ RO-82-W-1 cells/well were plated in 96 multiwell plates in regular medium containing 10% serum (FCS). 24 h later, the cell medium was removed and cells were incubated in starvation medium (1% FCS and 0.5 g/L glucose). Viability was determined by MTT assay (Thermo fisher, Milan, Italy) after 72 h following kit's instructions. For studies with TKIs, after 24 h with starvation medium, cells were stimulated with Lenvatinib, Sorafenib, Vandetanib and Cabozatinib at 1 or 10 µM. Viability was assessed after 72 h with MTT.

Western blot

Cells were lysed with CelLytic™ M reagent (Sigma-Aldrich, Milan, Italy). Proteins contained in the supernatant were quantified by Bradford assay (Sigma-Aldrich, Milan, Italy) using a standard curve prepared with BSA (stock concentration 2mg/ml) composed of 5 dilution points (250, 125, 50, 25 and 5 µg/ml). Fifty micrograms of proteins were mixed with 4X reducing SDS-PAGE sample buffer and denatured at 100°C for 10 min. Electrophoresis was carried out in SDS/10% polyacrylamide gel at 150 V. Proteins were then blotted onto nitrocellulose membranes at 390 mA for 90 minutes. The membranes were blocked with 5% milk in TBS with 0.05% Tween 20 (TBST) for 1 hour and incubated with the primary antibody (1:2000) diluted in TBST with 5% BSA overnight at 4°C. The membranes were thoroughly washed and then incubated with the secondary antibody (1:2500) diluted in TBST containing 5% milk for 1 hour at room temperature. All blots were developed using the ECL plus kit from Amersham following the manufacturer's protocol and captured with ImageQuant LAS 500 (GE Healthcare, Milan, Italy). OD arbitrary units were calculated using the ImageJ software subtracting the background from each measures.

Statistical Analysis

All data will be calculated as mean±SD of at least three independent experiments. Statistical analysis will be performed with GraphPad Prism software version 5 using one-way ANOVA for multiple group comparison. P value <0.05 will be considered significant. For combined treatment, the cooperative index (CI) will be calculated as the sum of the specific cell deaths induced by the single agents divided by the specific cell death in response to the combination. CI values <1, $=1$ and >1 indicate a synergistic, additive or infra-additive effect, respectively.

Results:-

Genetic characterization of thyroid cancer cells

Cancer cells employed in this study were characterized for their genetic profile for the most common mutations involved in thyroid cancer development. As summarized in table 1, 8505c and BCPAP harbored the BRAF p.V600E mutation. TPC1 and RO-82-W-1 had RET/PTC1 rearrangement whereas TT medullary cell line carried the RET point mutations p.C634W and p.G691S. Cal62, Hth7 and C643 harbored the KRAS p.G12R, NRAS p.Q61R and HRAS p.G13R, respectively (Table 1).

Effect of starvation conditions on thyroid cancer cell growth

To verify whether culture conditions mimicking the metabolic consequences of fasting reduce cancer growth, thyroid cancer cells were exposed for 72 hours to 0.5 g/L glucose and low serum (1%) (starvation medium). We tested cancer cells of different histotypes including anaplastic (8505c, Cal62, Hth7 and C643), papillary (TPC1 and BCPAP), follicular (RO-82-W-1) and medullary (TT). As shown in Figure 1, cell growth (evaluated by MTT test) was significantly reduced in all cell types with an inhibition rate ranging from approximately 30% to 80%. The effect on cell proliferation was not linked to a specific genetic alteration.

Combination of fasting condition and TKI treatment on thyroid cancer cell growth

To explore whether fasting condition would potentiate the anti-proliferative effect of TKIs in current clinical use, we treated cells with 1 (not shown) or 10 µM of Lenvatinib, Sorafenib, Vandetanib and Cabozantinib for 72 h after a pre-incubation of 24 h with starvation medium. Data are reported in Figure 2. In anaplastic cancer cells (8505c, Hth7, C643 and Cal62), Lenvatinib did not affect cancer cell proliferation. The only inhibitory effect obtained was restricted to treatment with starvation. For papillary thyroid cancer, BCPAP were not affected by TKIs (both Lenvatinib and Sorafenib) and no differences were observed between Lenvatinib alone or in combination with starvation. The decrease observed with sorafenib + starvation against sorafenib alone, was probably due to the only effect of starvation. In TPC1, cell growth was significantly reduced by TKIs (Lenvatinib and Sorafenib) and starvation alone with a similar extent. In addition, starvation significantly potentiate TKI activity. For the follicular thyroid cancer (RO-82-W-1), cell growth was decreased by treatment with Lenvatinib and Sorafenib. The inhibitory effect of TKIs was significantly potentiated by fast mimicking medium especially for Sorafenib. Medullary thyroid cancer TT cells, were affected by Sorafenib, Vandetanib and Cabozantinib. For the treatment with Vandetanib no advantage was acquired by the presence of starvation. On the contrary, the combined treatment of starvation with Sorafenib or Cabozantinib increased the anti-proliferative effect of TKIs.

Combined effect of TKIs and starvation on ERK1-2 phosphorylation

Based on the above results, we selected TPC1, TT and RO-82-W1 (all harboring a defect on RET receptor) cells for further experiments. Anaplastic thyroid cancer cells and BCPAP were excluded as TKIs had no effect on cell growth of these cells. As pointed out in the introduction, TKIs are proposed for the treatment of refractory thyroid cancer as they inhibit signaling involved in thyroid oncogenes such as MAPK pathway. We, then measured ERK1-2 phosphorylation by western blot after 1, 6 (not shown), 12 (not shown) and 24 hours of treatment. As shown in figure 3, TKIs significantly reduced ERK1-2 activation after 1 h in all cell types. Starvation alone was effective starting from 6-12 h depending on cell type (not shown) and its effect last also at 24 hours of treatment (figure 3). Prolonged (24 h) exposure to TKIs resulted in an improvement of ERK1-2 phosphorylation indicating that cells can activate alternative mechanisms to bypass TKI inhibitory activity. In all cases (except for RO-82-W1 treated with Lenvatinib), the combination of starvation and TKIs was the most valuable at maintaining ERK1-2 phosphorylation inhibition at 24 hours (Figure 3).

Discussion:-

TKIs can be considered the first line therapy for poorly differentiated, anaplastic, and advanced medullary thyroid carcinoma, which are not responsive to conventional therapy (1-2). Several trials have demonstrated that TKIs can extend the progression free survival (PFS) in refractory thyroid cancer patients (16-18) because of their effects on the genetic pathways involved in thyroid oncogenesis such as MAPK and PI3K-AKT. However, TKIs present dose-related side effects and patients who are initially responsive to treatment eventually develop resistance with progression of disease and death (19-21). Diet is an environmental factor with potential to influence cancer onset by shaping the epigenome (22). In addition, many types of cancer cells cannot adapt to depletion of nutrients and react to starvation promoting signaling pathways and protein synthesis resulting in a metabolic imbalance and death due

to oxidative stress (8, 11, 23). Cycles of fasting have been demonstrated to reduce tumor growth and to synergize with chemotherapy in *in vitro* studies (8, 11, 23). Using short-term fasting regimens to improve the efficacy of current and emerging cancer therapies remains a relevant research area. Many clinical trials (NCT01304251, NCT01175837, NCT00936364, NCT01802346, NCT02126449) are actually investigating the effects of fasting or fasting-mimicking diets in patients treated with chemotherapy for breast, prostate and other solid malignant neoplasm. Results are encouraging and demonstrated that cycle of fasting are well tolerated and associated with reduction of chemotherapy side effects (10, 24-26). Similarly, Caffa et al. (11) examined the role of fasting in association with TKIs in cancer cells (breast and colorectal) and in xenografts models and demonstrated that fasting mimicking diets increased TKIs anticancer activity by strengthening the MAPK inhibition. Due to the fact that TKIs are the elite treatment for thyroid cancer refractory to conventional therapies, we explored the possibility that starvation can support the efficacy of TKIs on thyroid cancer cells. First of all, we tested the effect of starvation alone on different thyroid histotypes including anaplastic, papillary, follicular and medullary. Cell growth was significantly reduced in all cell types with an inhibition rate ranging from 30% to 80%. We, also, evaluated the combination of fasting and TKIs already approved in the treatment of thyroid cancer and we reported a synergistic activity between TKIs and fasting for papillary, follicular and medullary thyroid cancer but not for the anaplastic. In addition, in agreement with the literature (11), in TPC1, TT and RO-82-W1 cells, starvation in addition with TKIs was the most successful at maintaining ERK1/2 phosphorylation inhibition. The major limit of this study is to rely only on in vitro experiments, thus studies on in vivo models are necessary to confirm the efficacy of TKIs administered under starvation conditions in thyroid cancer. However, these results support the introduction of short-term fasting regimens in the management of refractory thyroid cancer and hopefully patients with advanced thyroid carcinomas will be enrolled in future in specific clinical trials investigating this aspect.

Acknowledgements:

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Table 1:-Genetic profile of thyroid cancer cells

Cell line		BRAF p.V600E	RET/PTC1	RET p.C643W/p.G691S	HRAS p.G13R	NRAS p.Q61R	KRAS p.G12R	Pax8-PPARγ
ATC	8505c	+	-	-	-	-	-	-
	Cal62	-	-	-	-	-	+	-
	Hth7	-	-	-	-	+	-	-
	C643	-	-	-	+	-	-	-
PTC	TPC1	-	+	-	-	-	-	-
	BCPAP	+	-	-	-	-	-	-
FTC	RO-82-W-1	-	+	-	-	-	-	-
MTC	TT	-	-	+	-	-	-	-

Figure legends

Figure 1: Cell proliferation evaluated by MTT assay for thyroid cancer of different histotypes treated or not with starvation medium. Data are expressed as percentage of survival. Starvation= 0.5 g/L glucose and 1% serum. *=p<0.05, **=p<0.01, ***=p<0.001 by Anova test with Bonferroni post test. Experiments were repeated three times.

Figure 2: Cell proliferation evaluated by MTT assay for thyroid cancer of different histotypes treated or not with starvation medium in the presence of TKIs (10 µM). Data are expressed as percentage of survival (triplicates). Starvation= 0.5 g/L glucose and 1% serum. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001 by Anova test with Bonferroni post test.

Figure 3: OD arbitrary units for ERK1-2 phosphorylation after 1 or 6 h in PTC1, RO-82-W1 and TT cells treated or not with starvation medium in the presence of TKIs (10 µM). Experiments were run in triplicate. A representative blot is shown for each experimental point. Starvation= 0.5 g/L glucose and 1% serum. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001 by Anova test with Bonferroni post test.

Fig. 1

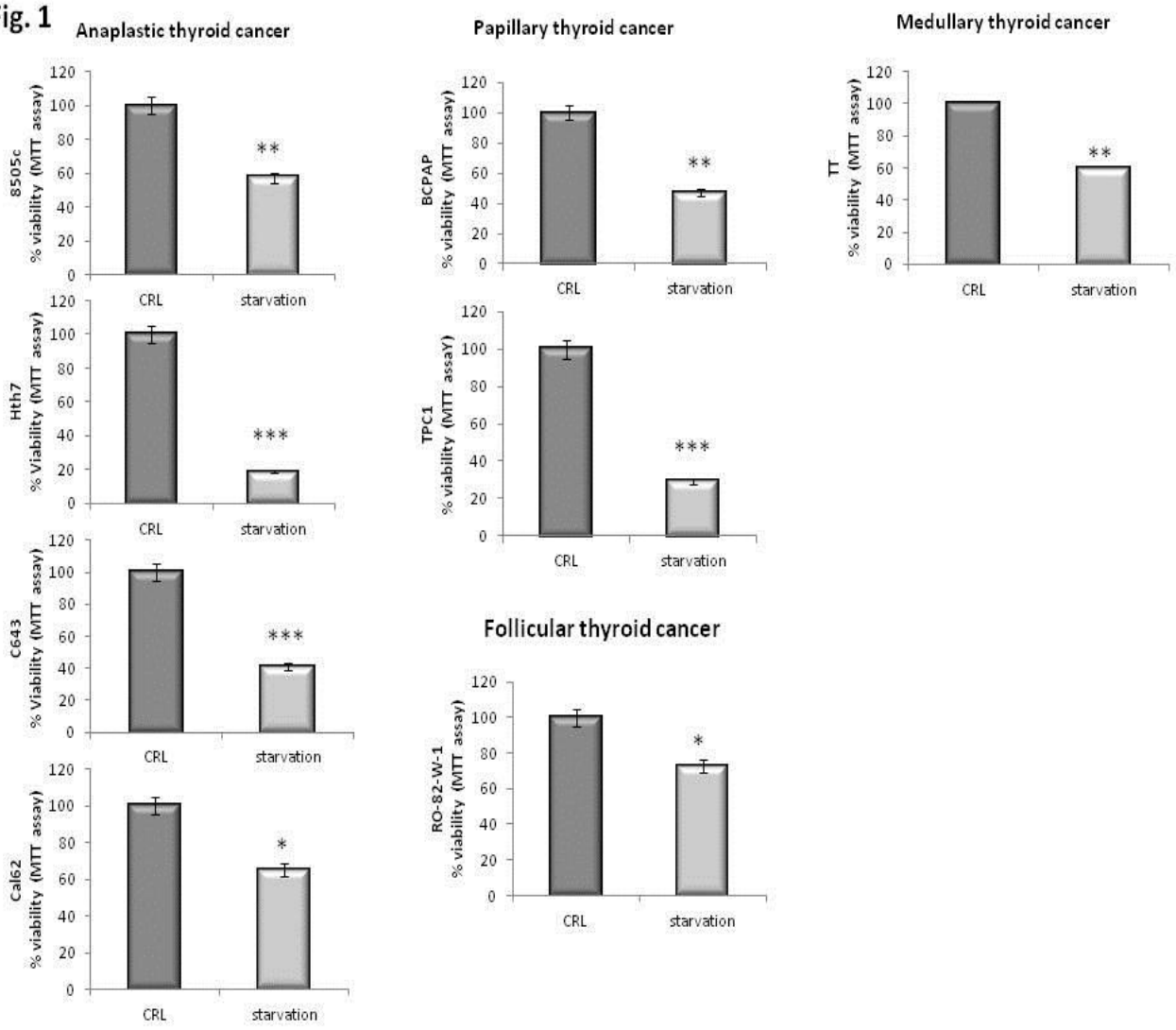
Anaplastic thyroid cancer
Papillary thyroid cancer
Medullary thyroid cancer
Follicular thyroid cancer
8505c % viability (MTT assay)
Hth7 % Viability (MTT assay)
C643 % Viability (MTT assay)
Cal62 % viability (MTT assay)
BCPAP % viability (MTT assay)
TPC1 % viability (MTT assaY)
TT % viability (MTT assay)
RO-82-W-1 % viability (MTT assay)
CRL
starvation
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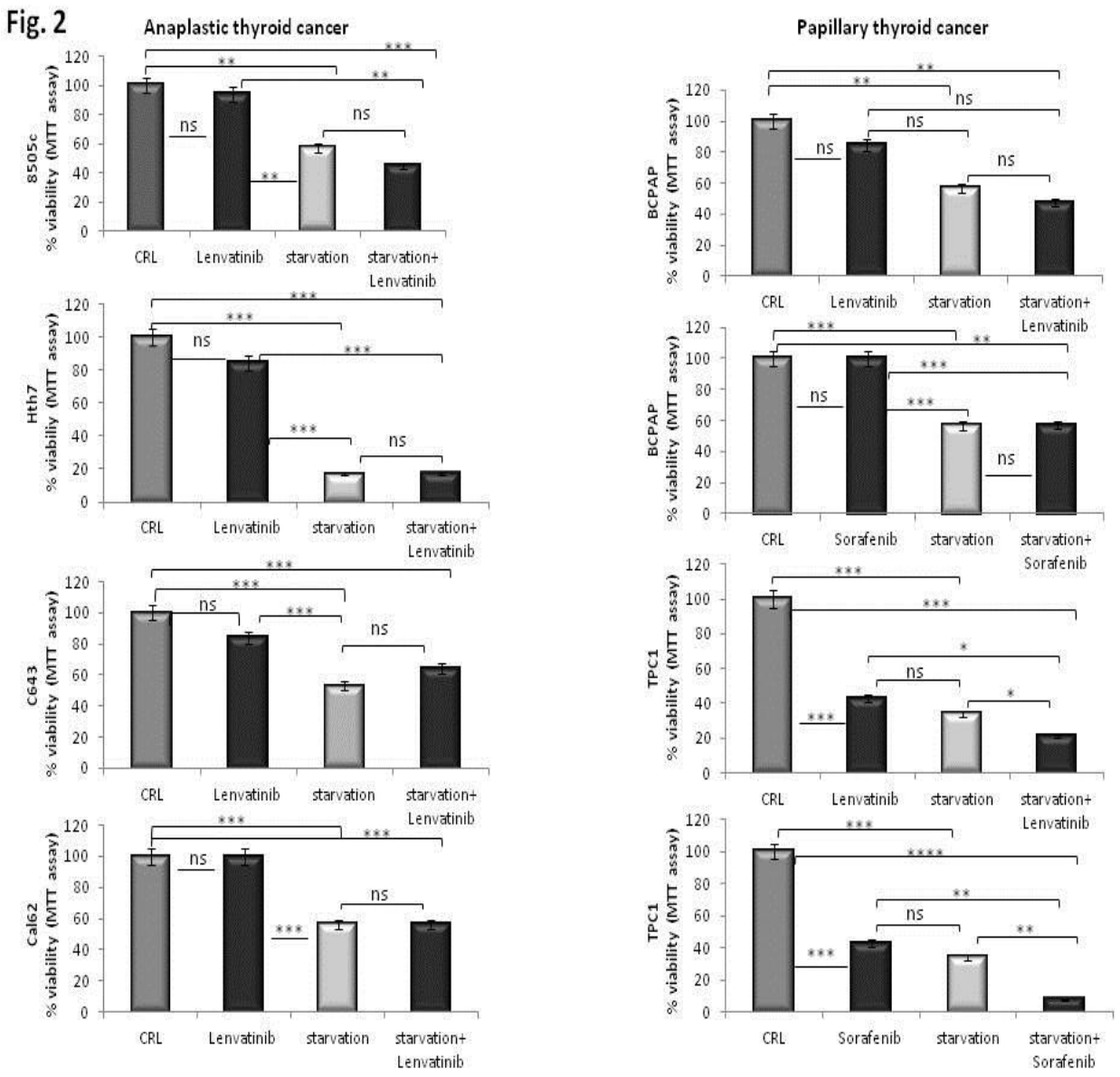
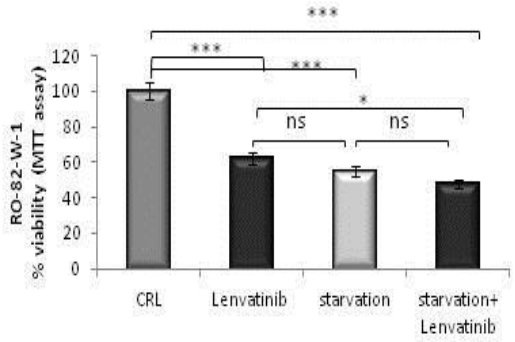
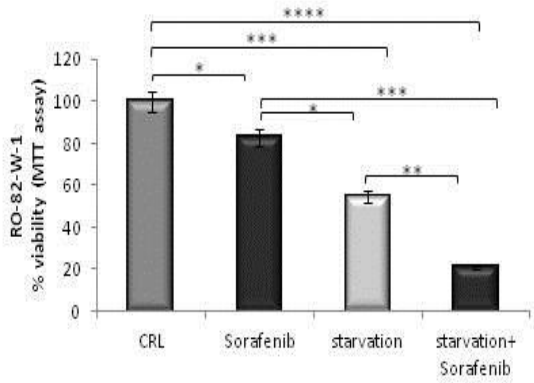
Fig. 2

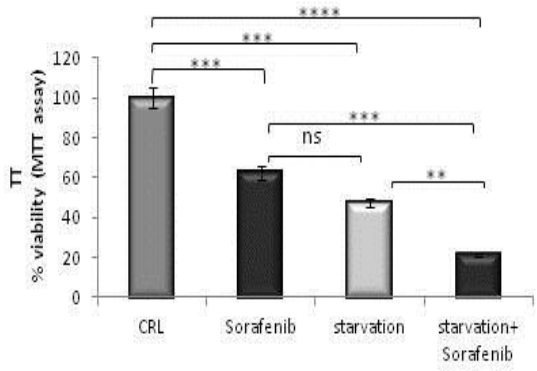

Anaplastic thyroid cancer
Papillary thyroid cancer
8505c
Hth7
C643
Cal62
BCPAP
TPC1
% viability (MTT assay)
CRL
Lenvatinib
Sorafenib
starvation
starvation+ Lenvatinib
starvation+ Sorafenib
ns

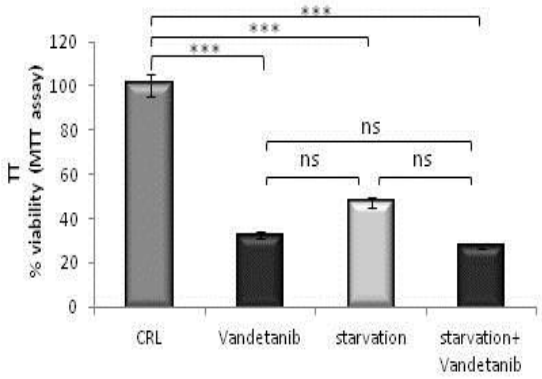
Follicular thyroid cancer





Medullary thyroid cancer





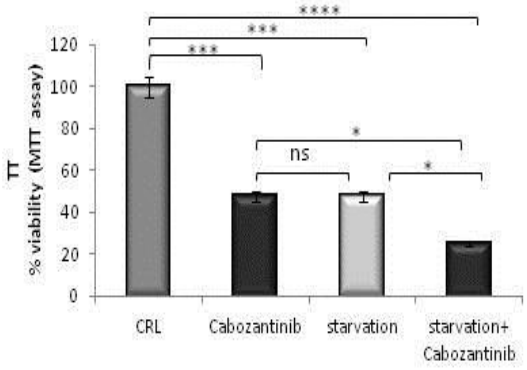
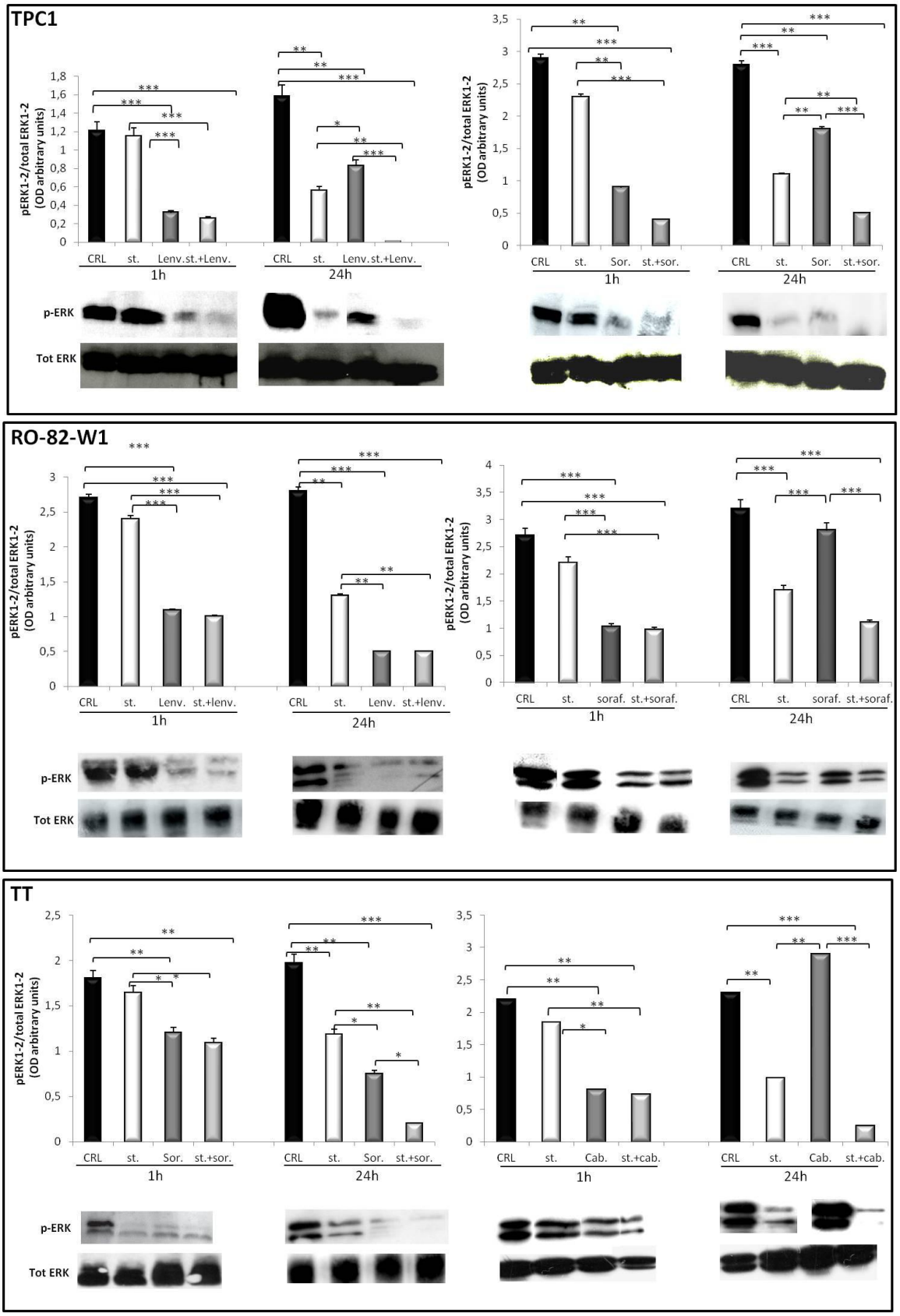


Fig. 3


TPC1
pERK1-2/total ERK1-2 (OD arbitrary units)
CRL
st.
Lenv.
st.+Lenv.
1h
24h
Sor.
st.+sor.
p-ERK
Tot ERK
RO-82-W1
soraf.
st.+soraf.
TT
Cab.
st.+cab.

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