INVESTIGATION OF CETUXIMAB RESISTANCE IN HEAD AND NECK SQUAMOUS CELL CARCINOMAS

FINAL REPORT

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1 BACKGROUND:

Squamous cell carcinomas of the Head and Neck region (HNSCCs) are the sixth most abundant malignancies, responsible for over 450.000 deaths annually [1]. Patients with recurrent/metastatic tumors receive first line combination chemotherapy as palliative treatment. This, according to the EXTREME protocol, involves six cycles of cisplatin/carboplatin and 5-fluorouracil treatment, alongside with the targeted treatment against the Epidermal Growth Factor Receptor (EGFR): cetuximab [2]. After six cycles, cetuximab monotherapy continues until progression. This combination regimen proved to be superior to monotherapies, however, response rates are low, and resistance often leads to early progression. Therefore, understanding (and avoiding) the mechanism of resistance is an urgent need. Among other factors (tumor hypoxia, tumor immunoevasion, tumor microbiome), the extracellular alterations of the transmembrane receptor EGFR were reportedly abundant in HNSCC and were suspected to contribute to therapy failure [3]. Also, alternative pathways of cellular survival and proliferation signaling, such as PI3K-AKT-mTOR pathway, or c-MET receptor based signaling was suspected to be involved [4]. However, the results published were unclear whether the changes in the receptor or alternative signaling might be important in cetuximab-resistance. Clarification of the problem might be of vital importance for many patients.

2 RESULTS:

2.1 EGFR, and cetuximab resistance in HNSCC

The problem implied the accurate screening of EGFR alterations in experimental systems and in clinical HNSCC samples as well. We found that, with huge variance among published data, the multi-exon deletion resulting truncated receptor variant (EGFR vIII), and the single nucleotide missense polymorphism (EGFR R521K) were reported to be abundant in HNSCC patients. Therefore, we performed targeted PCR of the crucial areas. For EGFR vIII detection, we used primer sequences for EGFR vIII: 5'-GCT CTG GAG GAA AAG AAA GGT AAT TAT and 5'- ACG CCG TCT TCC TCC ATC T. Primer sequences for wild-type EGFR detection were: 5' – TAC CTA TGT GCA GAG GAA TTA TGA TCT TT and 5' – CCA CTG TGT TGA GGG CAA TG. Shockingly, our results showed that only one patient (of the total of 95) was harboring EGFR vIII, therefore it was ruled out from possible resistance-drivers. Our data, alongside with another study, contradicts reports which claimed high frequency of EGFR vIII variant among HNSCC patients, and calls for clear presentation of sequencing data.

Parallel with EGFR vIII, we also screened HNSCC for EGFR R521K mutation. Among all 95 patients examined, 49 (51.6%) were found to harbor at least one allele of EGFR R521K. This enabled the possibility of a clinical relevance of the mutation on the high rate of cetuximab-resistant tumors.

Alongside with patient genotyping, we also used four well-known HNSCC cell lines - PE/CA PJ41 (mentioned as PJ41 in this report), PE/CA PJ15 (mentioned as PJ15), Cal-27 and FaDu. The cells were all negative for EGFR vIII. As for R521K polymorphism, PJ41 and Cal-27 were found homozygous wild-type, while PJ15 and FaDu were heterozygous for EGFR R521K, serving as good preclinical models for the investigation of cetuximab efficacy in EGFR wild type and EGFR R521K tumors.

First, we checked whether the cell models express different quantity of EGFR, therefore we quantified the receptor expression with immunolabeling of the cells with monoclonal antibody mAb528 and cetuximab, evaluated by flow cytometry. The results showed that no pattern was associated with R521K status (PJ41 and PJ15 cells had moderate EGFR expression, while Cal-27 and FaDu had higher receptor levels).

We started to investigate direct effects on cell proliferation and activation. We found, that up to 100 uM concentration, no toxic or antiproliferative effects were detectable on the HNSCC cells. Results are visualized on **Figure 1**.

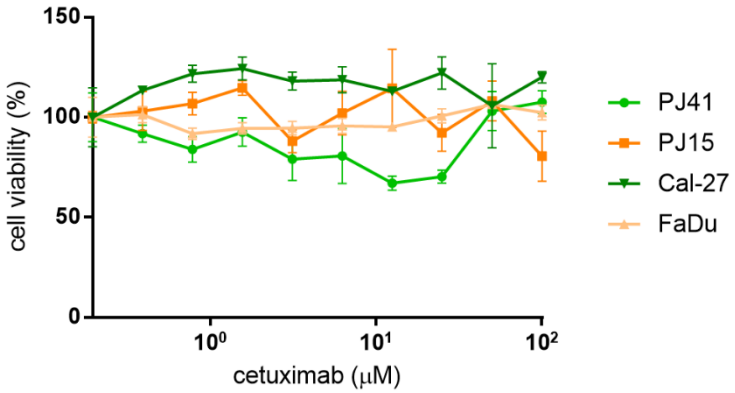

Figure 1. Proliferation of HNSCC cells following 72-hour treatment with cetuximab.

We also examined the EGFR phosphorylation to see whether EGFR R521K causes impairment of phosphorylation inhibition. Measurement of protein levels of total and phosphorylated EGFR showed that at all common sites of EGF-dependent activation of the receptor, phosphorylation was efficiently inhibited in all cell lines, showing that the intracellular effects are alike, regardless of R521K status (**Figure 2**).

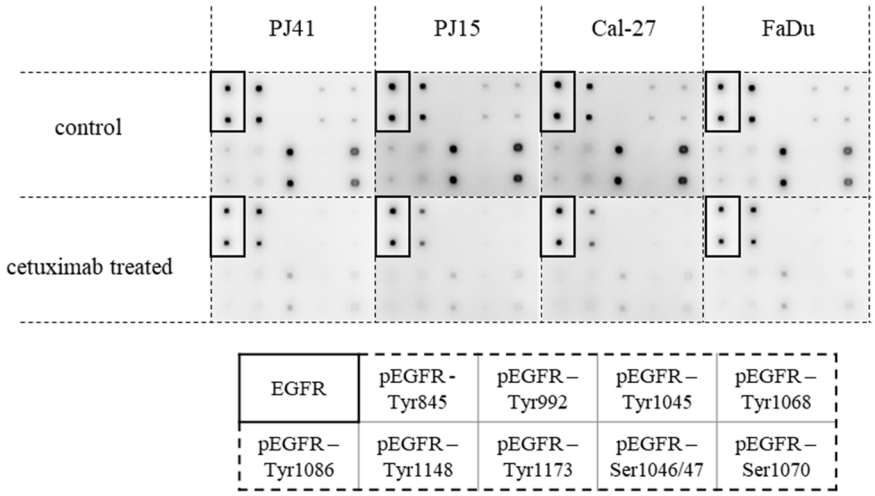

Figure 2. EGFR and phospho-EGFR protein expression.

In-depth analysis of activation of EGFR and the possible parallel signaling driven by Hepatocyte Growth Factor Receptor (c-MET), we checked the activation of the two receptors using immunocytochemistry on PJ41 and PJ15 cells, as seen in **Figure 3.**

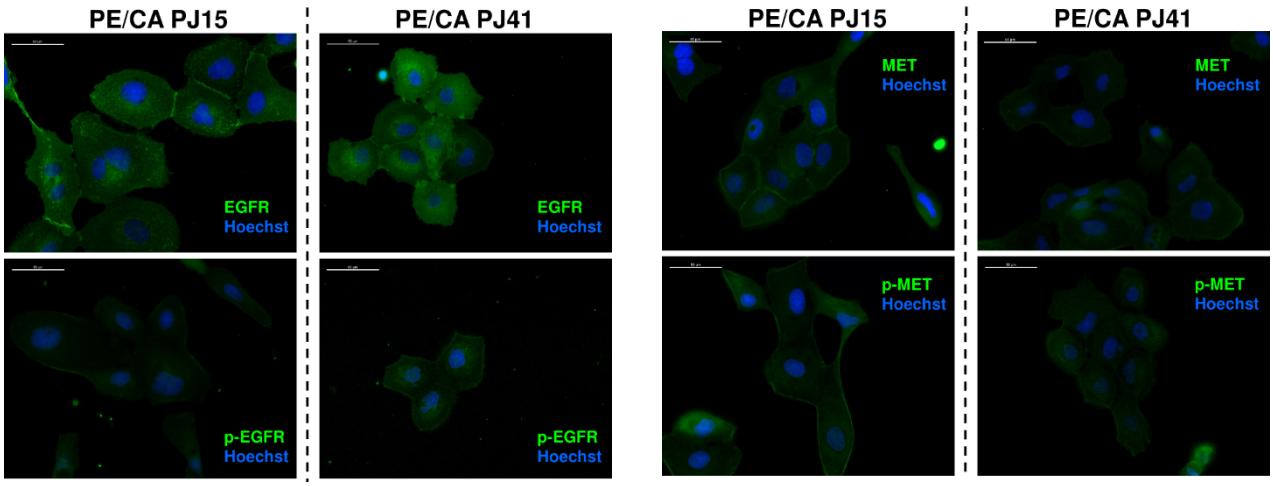

Figure 3. Total and phosphorylated EGFR and MET expression on HNSCC cells.

While *in vitro* tests of cetuximab-tumor cell relation showed no significant differences, we were also measuring antibody-dependent cellular cytotoxicity (ADCC) using Electric Cell-substrate Impedance Sensing system to follow up real-time cell viability of tumor cell cultures alone and in cocultures with human NK-cell derived CD16.176V.NK-92 cells. Compared to the control cocultures, cetuximab enhanced the tumor cell toxicity more effectively in EGFR wild type PJ41 and Cal-27, while it was less effective in EGFR R521K harboring PJ15 and FaDu. This difference emphasized that EGFR extracellular alteration might effectively influence cetuximab-mediated HNSCC tumor cell elimination.

To test this phenomenon, we used multiple *in vivo* xenograft models to test tumor growth and metastatic activity. Cetuximab treatment was extremely effective in EGFR wild type PJ41 and Cal-27 models, causing about complete remission of the tumors within three weeks. Interestingly, it was also effective in EGFR R521K harboring FaDu model, although it could not reduce the tumor volumes, it successfully prevented rapid tumor growth. In PJ15 xenograft model, cetuximab treatment had only modest effect, not being able to suppress tumor cell proliferation (results summarized on **Figure 4**).

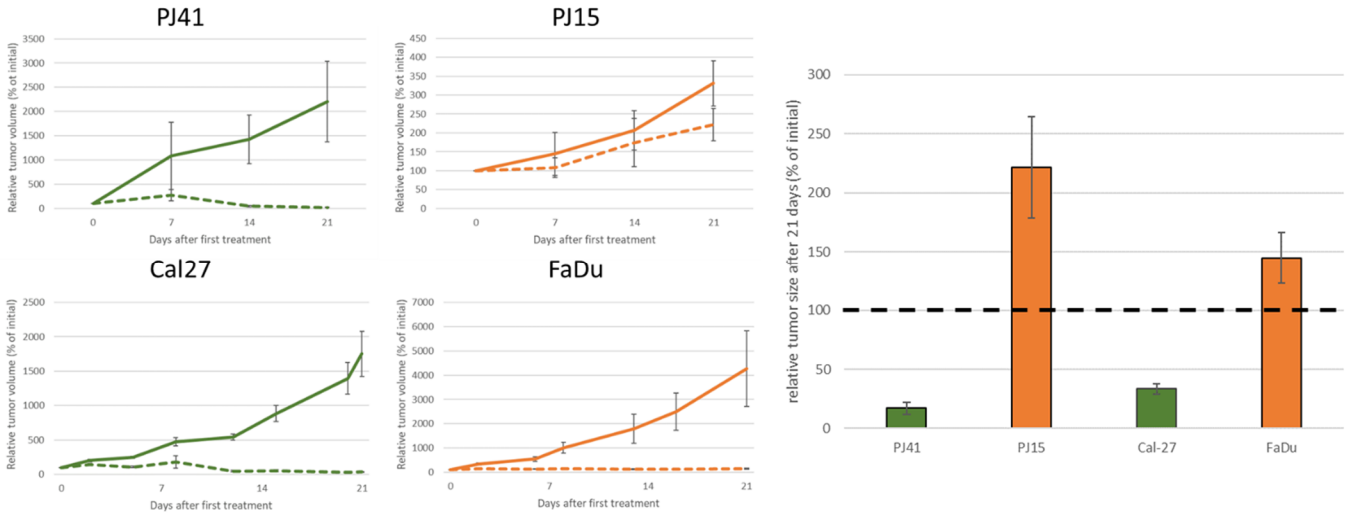

Figure 4. Tumor growth and final relative tumor sizes following cetuximab therapy of HNSCC xenografts.

Besides cetuximab efficacy, we measured the antitumor effect of MET inhibitor SU11274 as an attenuator of possible parallel signaling for tumor escape in case of EGFR inhibition. MET inhibition was effective against both primary tumor growth and metastasis formation in case of the R521K PJ15 model, while, surprisingly, it had no such effect against EGFR wild type PJ41 growth or spreading. This important finding might shed light to the possible use of c-MET inhibition as future strategy against cetuximab-refractory head and neck cancers.

Our human HNSCC models were engrafted into SCID mice, a commonly used host strain lacking functional T- and B-cells. However, these mice still express activity of the cells of the innate immune response, as NK cells and macrophages. The previously performed *in vitro* ADCC experiments also emphasized the role of NK-cell activity in the mechanism of action of cetuximab. To spot the crucial cells, we performed a combined therapy experiment of cetuximab and established inhibitors of NK-cell activity and macrophage fuction. As expected, immune activity inhibition further enhanced the growth of the tested Cal-27 xenografts, while, surprisingly, these inhibitors failed to reduce the success of cetuximab against the tumors. This result was a warning signal that cetuximab antitumor effect might be more complex than a simple antibody-mediated elimination by immune cells. The combination treatment of cetuximab (CET), NK-cell inhibitor (GM1) and macrophage inhibitor (CL) could be followed on **Figure 5.**

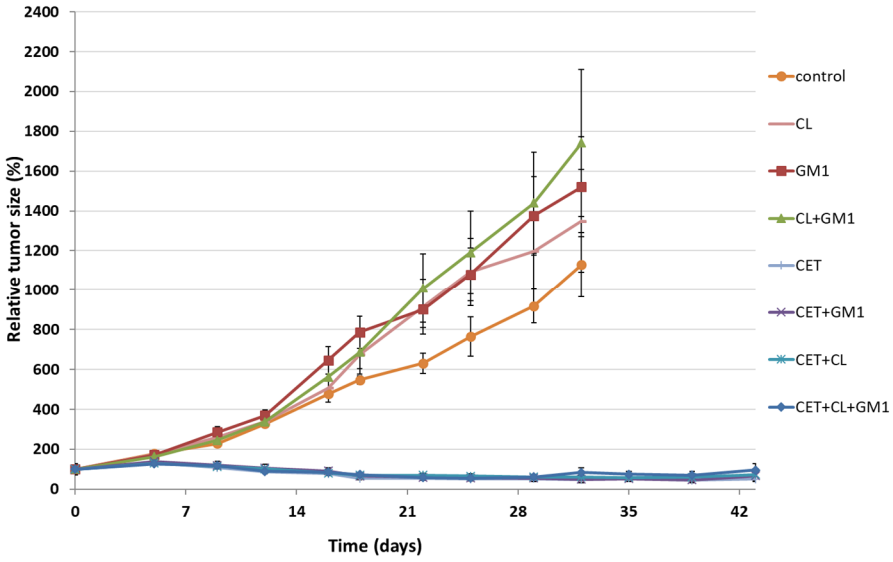

Figure 5. HNSCC xenograft growth by treatment with anti-EGFR cetuximab (CET), NK-cell inhibitor (GM1) and macrophage inhibitor (CL).

While preclinical models offer a good starting point and a controllable environment to test chemotherapy efficacy, our initial motivation was to find possible mechanisms of clinical resistance or sensitivity against cetuximab in HNSCC patients. Therefore, we analysed all EGFR genotype and clinicopathological variables, correlating EGFR R521K status with EGFR intron 1 CA repeat number, immunohistological analysis of intratumoral immune cells, and therapy outcome.

The total number of patients analysed was 95. However, since the formerly used EPTF combination therapy (including additional docetaxel treatment) was discontinued, we focused on the 63 patients available receiving EPF therapy according to the EXTREME protocol. EGFR R521K genotypes of the cohort is summarized in **Table 1.** For later analyses, we excluded 4 patients confirmed to be positive for HPV infection, further investigations were performed on the cohort of 59 comparable patients.

Total	EGFR wt	EGFR R521K heterozygous	EGFR R521K homozygous
63	29 (46%)	25 (40%)	9 (14%)

Table 1. Number and proportion of patients with various EGFR R521K genotypes.

As EGFR intron 1 CA repeat number was proved to be associated with decreased transcriptional activity of EGFR gene, we investigated whether CA repeat number correlates with EGFR R521K geotype. As shown in **Figure 6**, there was no CA repeat (therefore protein expression suppression) difference among patient groups with various EGFR R521K status.

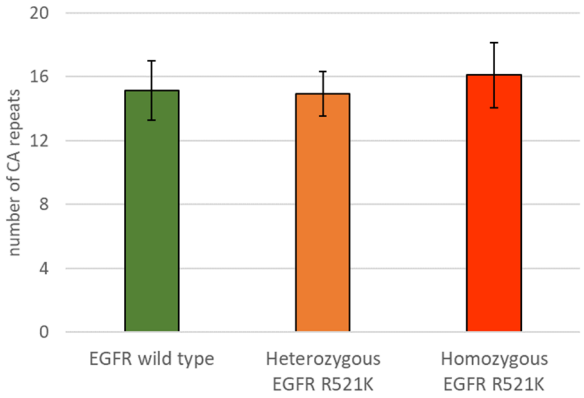

Figure 6. Quantification of EGFR intron 1 CA repeats among EGFR R521K groups.

As our *in vitro* and *in vivo* models raised the possibility of the importance of immune cells in cetuximab response, we performed multiple immunohistochemical analyses of HNSCC FFPE samples to quantify cells positive for CD16 (FcγIII – neutrophils, macrophages, NK-cells), CD68 (macrophages), or NKp46 (NK cells). The immune cell infiltration values were not different in EGFR wild type and EGFR R521K (heterozygous and homozygous polymorphic) patients, as **Figure 7** shows.

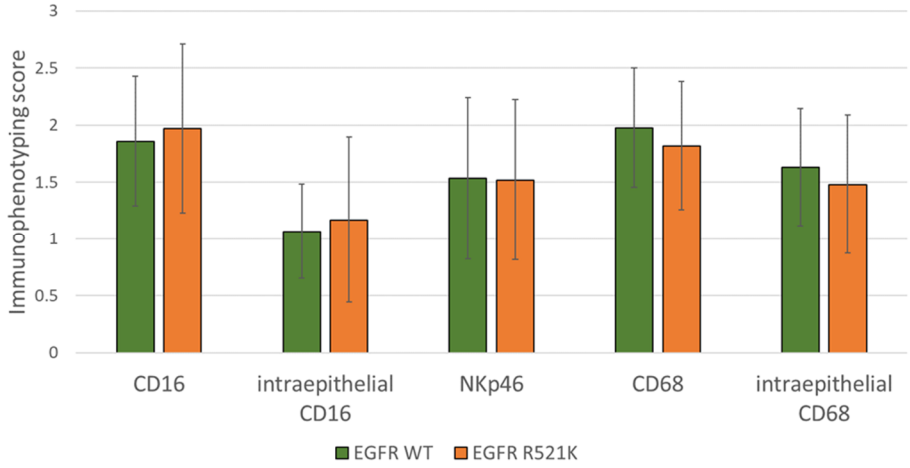

Figure 7. Immune cell infiltration of EGFR wild type and EGFR R521K HNSCC tumors

We further analysed clinical outcome of the patients, summarized in **Table 2.**

Patients	Total 59	EGFR wt 29 (49%)	EGFR R521K 30 (51%)	p
Men (n, %) Women (n, %)	44 (75%) 15 (25%)	26 (90%) 3 (10%)	18 (60%) 12 (40%)	p<0.05
Age (median, years)	57	56	58	no correlation
Overall response rate (n, %)	40 (68%)	22 (76%)	18 (60%)	no correlation
Disease contol rate (n, %)	55 (93%)	28 (97%)	27 (90%)	no correlation
Progression free survival (median, weeks)	33	35	31	no correlation
Overall survival (median, weeks)	58	63	50	no correlation

Table 2. Clinicopathological features of EGFR wild type and EGFR R521K HNSCC patients

In-detail analysis of the above data showed that all clinical outcome values in our cohort were showing slightly better patient response, compared to the original clinical trial results of the EXTREME protocol. It might be due to population specificity, or some refinement in diagnostic and supportive methods.

Importantly, the overall response rate, disease control rate, progression-free survival and overall survival (for the latter two, see **Figure 8.**) were not significantly different between EGFR wild type and EGFR R521K patients. These surprising results are in contrast with our preclinical findings, and, importantly, contradict to a publication previously published in the journal Cancer Research, which raises awareness of the critical thinking over published data.

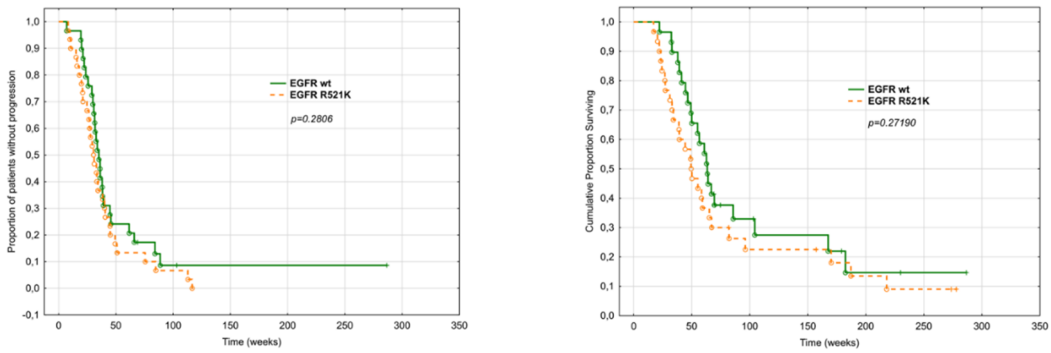

Figure 8. Progression-free (left) and overall (right) survival of HNSCC patients of different EGFR R521K status.

In summary, evaluating our clinical data and comparing it with other HNSCC cohorts, our point of view is that in a clinical setup, neither EGFR R521K nor EGFR vIII alterations are suitable to determine cetuximab therapy success. Of note, preclinical results of cetuximab monotherapy are hardly comparable with the complex effect of the combination chemotherapy in clinics. Therefore, our results do not support the future introduction of EGFR extracellular alteration analysis before cetuximab treatment indication (***Manuscript submitted***). Of great importance, our finding that cetuximab-resistant HNSCCs might respond well to c-MET targeting therapy, offers a new hope to overcome therapy failure [5].

2.2 Immune cell infiltration as predictive or prognostic factor of therapy

While EGFR R521K was not in close relation to tumor infiltrating immune cell presence, we succeeded to show that immunophenotype of the clinical HNSCC tumors were associated with survival data [6]. Furthermore, we published two studies on the importance of immune cell infiltration in malignant melanoma, too [7,8].

2.3 N-glycosylation, as a potential modifying factor of protein structure and antibody binding

We successfully applied the enzymatic reaction of PNGase F in order to characterize HNSCC cells and tumor sample receptror N-glycosylation, which was suspected to alter the receptor surface, thus potentially influencing cetuximab binding. Detailed method for sample preparation from both fresh and FFPE samples was established and published [9,10], however, in our clinical cohort, no characteristic changes were present for patient selection or therapy outcome prediction.

2.4 The role of EGFR-signaling in therapy resistance

Furthermore, we investigated different aspects of EGFR-related signaling pathways extensively. Mutations of KRAS [11], BRAF [12], role of MAPK and FAK signaling in Malignant mesothelioma [13,14], and role of Tks4 [15,16] were discussed.

2.5 Hypoxia-driven resistance

Tumor hypoxia effects on tumor cell motility and metastatic activity were extensively analysed, including the characterization of the effects in HNSCC cell PJ15 [17], pointing to the effect of hypoxia in increasing migratory and metastatic activity (often associated with therapy resistance), and details of the migration process were unveiled [18].

2.6 Targeted therapy by homing instead of antibody-binding: Cell Penetrating Peptides

Our collaborative works enabled the investigation of a different aspect of targeted therapy of tumor cells. Instead of inhibiting or immunolabeling the target moieties, peptide-drug conjugates were designed, produced and tested successfully to anchor and ingest the linked cytotoxic drug into the target cells [19–23].

2.7 Further aspects of chemotherapy resistance

Parallel works of our lab were focused on different important aspects of therapy resistance. The selective killing of MDR tumor cells via iron depletion [24], inhibition of angiogenic receptor tyrosine kinases[25], and novel selective inhibitor of FLT3 receptor [26] were measured and published recently.

3 REFERENCES:

*Note: references produced in connection with the project are highlighted as **bold**.*

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