Among non-small cell lung cancer (NSCLC) patients, the most common HER2 mutation is the exon 20 insertion mutation, Y772dupYVMA, which accounts for over 40% of all HER2 mutations in lung cancer. Trastuzumab deruxtecan (T-DXd), a HER2 antibody-drug conjugate (ADC), received FDA approval for the treatment of HER2 mutant NSCLC yielding a confirmed objective response of 52% and a median duration of response of greater than 9 months. Unfortunately, patients that initially respond to T-DXd will eventually acquire resistance, and the mechanisms of resistance as well as effective targeting strategies to overcome resistance are not yet elucidated. We generated Ba/F3 cells expressing the HER2 YVMA insertion mutation with acquired resistance to T-DXd by culturing cells in T-DXd until resistance occurred. T-DXd resistant cells retained expression of HER2, but were resistant to the payload, deruxtecan, as well as other topoisomerase inhibitors. T-DXd resistant cells exhibited loss of topoisomerase I, a previously reported mechanism of topoisomerase inhibitor resistance. T-DXd resistant cell lines were sensitive to payloads with alternate mechanisms of action including maytansine and likewise retained sensitivity to the HER2 ADC trastuzumab emtansine (T-DM1) which utilizes DM1 as a payload. Given that T-DXd resistant cells retained HER2 expression, we assessed whether they were sensitive to HER2 tyrosine kinase inhibitors (TKI). T-DXd resistant cells remained highly sensitive to HER2 TKIs including poziotinib, afatinib, and zongertinib. To investigate whether genomic alterations of HER2 could facilitate HER2 ADC resistance, we utilized the LentiMutate approach which employs an error-prone HIV-1 reverse transcriptase to produce a high frequency of mutations to identify resistance-associated mutations in cells treated with a HER2 ADC. Sequencing analysis revealed that resistant cells had an enrichment in point mutations within HER2 domain IV (D582N, F595C/S, E580K, C623Y), which includes the binding site of trastuzumab. To validate the impact of these mutations on HER2 ADC resistance, we generated cells expressing a HER2 exon 20 insertion in combination with the observed domain IV mutations. D582N and F695C/S co-mutations conferred resistance to T-DXd and T-DM1 but not to HER2 TKIs. Next, we generated a PDX model of acquired T-DXd resistance and observed that resistant tumors expressed a truncated form of HER2 which lacked the extracellular portion. These data demonstrate that resistance to T-DXd can be mediated by multiple mechanisms including loss of sensitivity to the ADC payload in which case ADCs bearing payloads with alternate mechanisms of action as well as HER2 TKIs retain anti-tumor cell activity. Moreover, resistance to trastuzumab-based ADCs can also be mediated by secondary mutations within domain IV of HER2 or loss of the extracellular terminal of HER2. However, these alterations do not diminish HER2 TKI activity.